Food and Waterborne Parasitology 4 (2016) 54–61

Contents lists available at ScienceDirect

Food and Waterborne Parasitology

journal homepage: www.elsevier.com/locate/fawpar

Cryptosporidium — What is it?☆

R.C. Andrew Thompson [a,*], Wan H. Koh [a], Peta L. Clode [b]

[a] School of Veterinary and Life Sciences, Murdoch University, South Street, Murdoch, WA 6150, Australia
[b] Centre for Microscopy, Characterisation, and Analysis, The University of Western Australia, Crawley, WA 6009, Australia

ARTICLE INFO

Article history:
Received 14 June 2016
Received in revised form 25 August 2016
Accepted 25 August 2016
Available online 27 August 2016

Keywords:
Cryptosporidium
Gregarine
Biology

ABSTRACT

Cryptosporidium is a ubiquitous enteric protozoan pathogen of vertebrates, and although recognised as a cause of disease in humans and domestic animals for over 50 years, fundamental questions concerning its biology and ecology have only recently been resolved. Overwhelming data now confirm that, like its close relatives, *Cryptosporidium* is a facultatively epicellular apicomplexan that is able to multiply in a host cell-free environment. These data must be considered in the context of the phylogenetic reclassification of *Cryptosporidium* from a coccidian to a gregarine. Together, they dictate an urgent need to reconsider the biology and behaviour of *Cryptosporidium*, and perhaps help to explain the parasite's incredible genetic diversity, distribution and host range. Improved imaging technologies have complemented phylogenetic studies in demonstrating the parasite's affinities with gregarine protozoa and have further supported its extracellular developmental capability and potential role as an environmental pathogen. These advances in our understanding of *Cryptosporidium* as a protozoan pathogen are examined with emphasis on how they may influence control strategies in the future.

Contents

☆ This paper is based on an invited presentation given by the authors in a symposium organized by the International Association for Food and Waterborne Parasitology as part of EMOP XII in Turku, Finland, 20-24 July, 2016.

* Corresponding author.
E-mail address: a.thompson@murdoch.edu.au (R.C.A. Thompson).

http://dx.doi.org/10.1016/j.fawpar.2016.08.004

1. Introduction

Cryptosporidium has been an enigma since it was first described by Edward Tyzzer in 1907 in the gastric glands of a mouse (Tyzzer, 1907). He placed it in the coccidian family Asporocystidae reflecting the lack of sporocysts in the oocyst (*i.e.* naked sporozoites) and what were presumed to be the possession of similar life cycle features (Levine, 1988). It is interesting when going back to Tyzzer's morphological description, how atypical it is for a coccidian, in particular the possession of an organ of attachment - a structure that has only recently been given the attention it clearly warrants in terms of considering *Cryptosporidium*'s true affinities.

For the next 70 years following Tyzzer's description, *Cryptosporidium* continued to be viewed as a curiosity. More species were described largely on the basis of host occurrence, but the parasite was always viewed as atypical. This was not only because of its oocyst and attachment organ, but also because of the ability of unshed oocyst to produce autoinfections, and the extracytoplasmic association with its host cell with endogenous developmental stages confined to the apical surfaces of epithelial cells, a characteristic now referred to as epicellular (Barta and Thompson, 2006; Clode et al., 2015; Thompson et al., 2005; Valigurová et al., 2007). However, these fascinating biological peculiarities were overshadowed by the serious public health consequences of opportunistic infections with *Cryptosporidium* that emerged in the 1980's, principally taking advantage of the weakened immune systems of AIDS patients (Checkley et al., 2014). This health emergency brought a sharp focus on the need for chemotherapeutics and quickly confirmed *Cryptosporidium*'s complete insensitivity to anti-coccidial drugs (Tenter et al., 2002; Thompson et al., 2005).

Cryptosporidium's direct life cycle is enhanced by the existence of resistant oocysts that are capable of extended periods of survival in the environment. Thus, apart from person to person transmission by the faecal-oral route, oocysts can be transmitted in water or contaminated food (FAO, 2014; Gajadhar et al., 2015). The emergence of cryptosporidiosis as an opportunistic infection put immense pressure on water utilities to ensure they provided *Cryptosporidium*-free water. The demands of water utilities for improved methods of surveillance to detect but also characterise isolates of *Cryptosporidium* was the main driver for research on the molecular epidemiology of *Cryptosporidium* infections (Cacciò et al., 2005; Thompson, 2003).

As a consequence, the biology and host-parasite relationship of *Cryptosporidium* have not received the attention they should have given the uniqueness of this organism. Recent developments in *in vitro* cultivation, life cycle propagation, phylogenetics, and imaging technologies have served to illustrate the need to re-evaluate many aspects of the biology and ecology of *Cryptosporidium* (Clode et al., 2015; Karanis and Aldeyarbi, 2011).

In this short review, we have tried to highlight the important developments over the last 100 years that have culminated in the recognition that *Cryptosporidium* is: a ubiquitous, pleiomorphic, facultatively epicellular gregarine protozoan, capable of extended existence in the environment, that is elusive, opportunistic and zoonotic with the *potential* to cause disease and death in humans and domestic animals.

2. Transmission — the importance of the environment

Direct transmission *via* the faecal/oral route is likely to be the most common form of transmission, whether zoonotic (see below) or direct person-to-person (FAO, 2014; Checkley et al., 2014). Waterborne outbreaks have been a major issue in the epidemiology of cryptosporidiosis throughout the world and a major financial burden for water utilities in developed countries. The problem has recently been shown to be exacerbated by the potential for biofilms to act as reservoirs of *Cryptosporidium* in which oocysts can not only be trapped and subsequently released into the water supply, but can also act as nutrient-rich environments

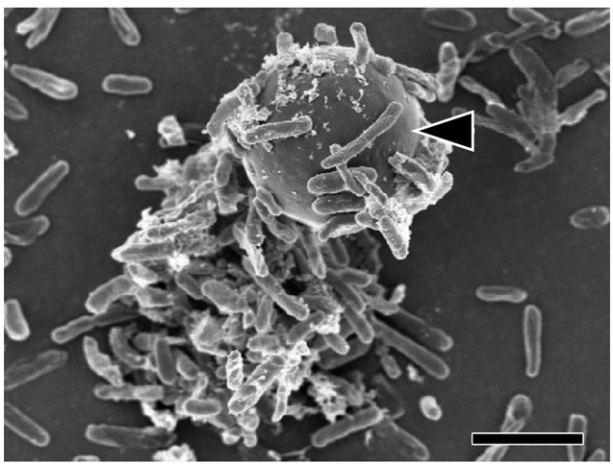

Fig. 1. Scanning electron micrograph of an oocyst of *Cryptosporidium* (arrowhead) within a *Cryptosporidium* – exposed *Pseudomonas aeruginosa* biofilm (see Koh et al., 2014 for methods). Scale bar = 3 μm.

in which the parasite can develop and multiply (Koh et al., 2013, 2014) (Fig. 1). Waterborne outbreaks involving drinking water or the contamination of public swimming pools have been, and are, principally an issue for developed countries (Thompson and Smith, 2011). Waterborne transmission of parasites in the developed world is therefore more likely to be the result of contamination, or a process failure within water utilities, industry, or in public places such as swimming pools.

Indirect transmission, where infection results through the mechanical transmission of oocysts on, for example, flies (Szostakowska et al., 2004) or other animals such as dogs or livestock, or by the contamination of food or local water sources poses a significant threat particularly in the developing world (Karanis et al., 2007; Nyarango et al., 2008; Smith et al., 2007; Thompson and Smith, 2011). The risk of infection is greater within rural environments than within urban areas; presumably because of the increased opportunity for both direct and indirect transmission to occur in areas with poor sanitation and higher contact rates with wildlife and domestic animal reservoirs of infection (Thompson and Smith, 2011). Poor hygiene is a crucial factor in enhancing the transmission of enteric protozoa such as *Cryptosporidium* (Smith et al., 2007; Thompson and Smith, 2011).

Foodborne transmission, as a result of agricultural practices, poor hygiene by food handlers or within households, is responsible for a significant number of infections with *Cryptosporidium* (FAO, 2014; Rose and Slifko, 1999; Thompson and Smith, 2011). However, linking infection to a contaminated food source is often difficult to determine, in many cases (Thompson and Smith, 2011). Small-scale outbreaks, where the point of initial contamination may be the result of poor hygiene by an individual resulting in localised foodborne transmission to family members or the immediate community, are likely to be extremely common particularly in the developing world (Thompson and Smith, 2011). Preliminary estimates derived from a literature review of published reports and case notes for *Cryptosporidium* occurrence, suggest that the number of cases of foodborne transmission resulting in infection in the WHO Eastern Mediterranean region (EMR) may be as many as 6 million cases annually and up to 27 million in the African region (Thompson and Smith, 2011). Transmission resulting from contamination of food or drink that leads on to larger scale infections, such as an outbreak of cryptosporidiosis in the USA in 1993 arising from the consumption of infected fresh-pressed apple cider, is a relatively much rarer event (Millard et al., 1994).

3. Impact on the health of humans and other animals — an opportunistic pathogen

Cryptosporidium is a significant cause of diarrhoeal disease, principally in humans and livestock throughout the world. The clinical impact of the parasite is greatest in hosts whose immune system is suboptimal as it is in infants and young livestock, the elderly, or those impaired by disease or stress (Checkley et al., 2014; Fletcher et al., 2012; Thompson et al., 2005).

In terms of control, there are different priorities in developed and developing countries. In the former, the need is for effective diagnosis and treatment for individuals and livestock, and the prevention of food and waterborne transmission, the latter being a significant economic issue for water utilities. In the developing world, the need is to lessen the burden of disease in those most at risk of infection, particularly children (Hotez et al., 2015; Savioli et al., 2006).

Cryptosporidium has gone through two phases of emergence as a cause of intractable diarrhoea and mortality. The first phase was during the AIDS crisis of the 1980s, the second is current and is likely to have a longer-term impact. *Cryptosporidium* is now recognised as a major contributor to diarrhoeal morbidity and mortality in children in the developing world, particularly Africa and South East Asia (Global Enterics Multisite Study (GEMS)1 and Mal-ED2 epidemiological studies) (Kotloff et al., 2013; Mbae et al., 2013). These studies suggest that *Cryptosporidium* may be responsible for a significant number of cases of moderate-to-severe diarrhoea in children under two living in these regions. Although nitazoxanide is used to treat *Cryptosporidium* infections its efficacy in those most at-risk, malnourished children and the immunocompromised, is limited (Checkley et al., 2014). The lack of therapeutic interventions is exacerbated by the lack of prophylactic measures including a vaccine, as well as the fact that people in the developing world where infection with *Cryptosporidium* is frequent, are commonly infected with several other species of intestinal and systemic parasites (Thompson and Smith, 2011). More serious in the long term in such populations is the emerging spectre of HIV. HIV-positive children in Tanzania were found to be almost eight times more likely to have *Cryptosporidium* than those who were HIV-negative (Tellevik et al., 2015). A study among Kenyan children also found this association (Mbae et al., 2013) and Tumwine et al. (2005) found that HIV-positive Ugandan children with persistent diarrhoea were 18 times more likely to have *Cryptosporidium* than those who were HIV-negative.

4. Problems of detection — molecular tools a revelation

In the clinical laboratory, whether dealing with samples from humans, domestic animals, or wildlife, there continues to be a need for rapid, sensitive and specific diagnostic tools that can guide appropriate therapy (Fletcher et al., 2012; Smith et al., 2006). Current laboratory methods that rely on microscopic examination of faecal samples for detecting *Cryptosporidium* oocysts have suffered from the problem of distinguishing the parasite from other faecal components of similar size and shape such as yeasts and algae. A number of staining techniques have been developed. Some such as those using malachite green provide reliable and consistent results, but many others suffer from problems of sensitivity, specificity, and variable results between laboratories (Elliot et al., 1999). However, microscopy cannot differentiate between the oocysts of *C. hominis*, zoonotic species, and the many other species and genotypes of *Cryptosporidium* since they are all morphologically indistinguishable in terms of size and it is only the larger oocysts of *C. andersoni* and *C. muris* that can be reliably distinguished from *Cryptosporidium* of public health significance (Smith et al., 2007). Even when combined with immunofluorescence, microscopy is relatively insensitive and prone to 'operator variability'. In contrast, a molecular approach offers greater sensitivity and specificity than traditional diagnostics reliant on microscopy.

As such, PCR-based procedures have dominated research on the diagnosis and detection of *Cryptosporidium* over the last 25 years, and in particular, because they have the added advantage of being able to provide information on the genotype or species of *Cryptosporidium* present in a clinical or environmental sample (Smith et al., 2006; Thompson and Ash, 2015). Such techniques are now replacing microscopy in many medical diagnostic laboratories, often in assays that are developed for the simultaneous detection of other enteric pathogens routinely screened for in clinical diagnostic laboratories, such as *Giardia* and *Blastocystis* (Cacciò et al., 2005). PCR-based tools have also proved to be of particular value in molecular epidemiological investigations by providing information on source of infection and the public health significance of isolates identified (Cacciò et al., 2005; Thompson et al., 2007).

The most commonly targeted gene used for characterising species of *Cryptosporidium* is SSU-rDNA (Xiao, 2010). In addition to the development of diagnostic assays, much research has also been concerned with the molecular epidemiology of water- and food- borne outbreaks and particularly the ability to detect and differentiate between those species commonly infecting humans (*C. hominis*, *C. parvum* (= *C. pestis*; Slapeta, 2011)). This research has increased understanding of the possible transmission routes from the environment and co-habiting animals such as companion animals and livestock (Fayer et al., 2000; Hunter and Thompson, 2005). It has also resulted in the identification of 'new' genotypes and the subsequent proliferation of new species and host ranges identified (Slapeta, 2013; Xiao and Fayer, 2008).

In addition to SSU-rDNA, molecular epidemiological investigations have identified additional genes of value as genotyping tools including the 70 kDA heat-shock protein (HSP70), the *Cryptosporidium* oocyst wall protein (COWP), and the internal transcriber region 1 (ITS-1) (reviewed in Thompson and Ash, 2015). For greater detail on possible transmission routes, intraspecific genotyping is required and the 60 kDA glycoprotein is commonly used to provide this level of discrimination (Lymbery and Thompson, 2011; Thompson and Ash, 2015).

5. Zoonotic potential — a recently recognized human pathogen

The first human case of *Cryptospordium* infection was described in 1976, and over the next 20 years considerable circumstantial evidence accumulated of zoonotic exposure associated with farms and farm animals, riding stables, animal manure, and contaminated water (Fayer et al., 2000). Many of these early reports drew attention to the association of human infection with exposure to infected livestock, particularly young cattle or sheep, and there was often evidence of secondary spread within households or play-groups following such zoonotic exposure (Casemore et al., 1997; Thompson, 2003). Although farm workers and visitors to farms were considered to have contracted cryptosporidiosis by direct contact, indirect zoonotic transmission of *Cryptosporidium* of livestock origin *via* water was considered at that time to be the most important zoonotic source of human infection (Thompson, 2003). However, up until the early 1990s such conclusions were often only circumstantial, with presumptions being made that run-off from pasture used for cattle, was the pre-disposing factor.

In 1991, analysis using restriction fragment length polymorphism (RFLP) revealed differences between *Cryptosporidium* of cattle and human origin (Ortega et al., 1991). In addition to confirming this result, subsequent molecular epidemiological studies demonstrated that humans were susceptible to infection with two genotypes of *Cryptosporidium*, one zoonotic (*C. parvum* (= *C. pestis*; Slapeta, 2011)), with cattle as its principal host, and the other host-specific for humans (*C. hominis*). This information was first put into an epidemiological context in 1997 in determining the source of contamination of the notorious Milwaukee outbreak (Peng et al., 1997), and subsequently in a series of outbreaks some of which were shown to be of zoonotic origin (Cacciò et al., 2005; Fletcher et al., 2012; Thompson, 2003).

Current evidence indicates that the main reservoirs of zoonotic *Cryptosporidium* remain livestock, with the potential transmission of *C. parvum* (= *C. pestis* (see Slapeta, 2011)), although other species, and genotypes, have been reported in humans but only occasionally (Slapeta, 2013). Susceptibility to infection with other host adapted species and genotypes is largely governed by the immune status of the host (Slapeta, 2013).

Interestingly, although cattle have been repeatedly implicated as sources of water-borne outbreaks, the application of genotyping procedures to the contaminating isolate(s) has often incriminated human effluent as the source (Hunter and Thompson, 2005; Thompson, 2003). The risk of infection appears greater within rural environments than within urban areas; presumably because of the increased opportunity for both direct and indirect transmission to occur in areas with poor sanitation and higher contact rates with domestic animal reservoirs of infection (Thompson and Smith, 2011).

6. Diversity — taxonomic issues impede progress?

The taxonomy of the genus *Cryptosporidium* has been controversial for many years with a number of taxonomic revisions that have seen species invalidated because descriptions were deemed inadequate in terms of morphological distinctness and/or concern that host occurrence was not worthy of species recognition (O'Donoghue, 1995; Slapeta, 2013). With the advent of molecular tools, the number of species has increased dramatically, the majority described on the basis of genetic distinctness and host occurrence. In most cases, there are minimal morphological characters to distinguish species of *Cryptosporidium*. Slapeta (2013) drew attention to the fact that in the last decade there was approximately one new species named each year, and 10 species proposed for 2004–2013. The most recent review listed 30 species but in addition to species that have been recognised as a result of surveys of humans and domestic animals there is growing evidence of numerous genotypes, identified in wildlife and in environmental samples (Appelbee et al., 2005; Oates et al., 2012; Slapeta, 2013). Given that *Cryptosporidium* is a gregarine we can expect

the number of species and genotypes to grow considerably since gregarines are considered to be the most diverse group of protozoa (see below).

7. Coccidial relationship challenged

Significant observations and research findings that have influenced opinion concerning *Cryptosporidium* being placed within the Gregarines are summarised in Table 1. Although believed for many years to be coccidia, species of *Cryptosporidium* were always considered to be atypical for a number of reasons (see Introduction). In addition to lacking key morphological structures such as sporocyst, micropyle, and polar granules, (See Table 2.)

a critical observation, although largely overlooked at the time, was a report of serological cross-reactivity with *Monocystis*, a gregarine (Bull et al., 1998). This relationship was reinforced when SSU-rDNA sequencing demonstrated that *Cryptosporidium* is more closely related to gregarines (Carreno et al., 1999). Most recently, Cavalier-Smith (2014) undertook a revision of gregarine higher classification, and the evolutionary diversification of sporozoa on the basis of gregarine site-heterogeneous SSU-rDNA trees. This has firmly placed *Cryptosporidium* within the gregarines, demonstrating that some 'eugregarines' and all 'neogregarines' are closely related to *Cryptosporidium*. Cavalier-Smith (2014) established a new subclass, the Orthogregarinia for *Cryptosporidium* and other closely related gregarines, with *Cryptosporidium* in its own subclass, the Cryptogregaria; defined as comprising epicellular parasites of vertebrates possessing a gregarine-like feeder organelle but lacking an apicoplast.

In addition to the 'molecular' evidence, *Cryptosporidium* shares many biological features with gregarines, including its epicellular location, connection to the host cell *via* a myzocytosis-like feeding mechanism, heterogeneity of trophozoite cell shape, and other structural similarities (Aldeyarbi and Karanis, 2016; Barta and Thompson, 2006; Borowski et al., 2008, 2010; Clode et al., 2015; Valigurová et al., 2007, 2008). The gliding movements seen in different stages of *Cryptosporidium* is a behavioural feature similar to the gliding motility exhibited by gregarines (Borowski et al., 2008, 2010; Sibley, 2004; Valigurová et al., 2013). The ability to observe the life cycle and development of *Cryptosporidium* in *in vitro* culture has made an important contribution to recognising *Cryptosporidum*'s gregarine similarities, not only by demonstrating previously unrecognised stages in the life cycle, incredible developmental plasticity and the occurrence of syzygy, but also the fact that *Cryptosporidium* is not an obligate epicellular parasite. *Cryptosporidium* has been shown to have the capacity to multiply epicellularly and extracellularly, again reflecting the fact that *Cryptosporidium* is closely related to gregarine protozoa (Hijjawi et al., 2004; Karanis et al., 2008, Koh et al., 2013, 2014; Rosales et al., 2005), which can also multiply by either means (Leander and Ramey, 2006).

The latest imaging technologies have played a major role in determining *Cryptosporidium*'s structural relationship with the gregarines but also how they share important similarities in their host parasite relationships (Clode et al., 2015; Koh et al., 2013, 2014). Most recently, *Cryptosporidium* has been shown to survive, multiply and develop in biofilms, salvaging nutrients from their nutrient-rich environment (Koh et al., 2014). Oocysts of *Cryptosporidium* have long been known to become trapped in biofilms where they can collect and then enter the water source in large numbers as a result of sloughing of the biofilm (Angles et al., 2007; Howe et al., 2002; Searcy et al., 2006). However, the fact that the parasite can excyst, develop, and multiply in biofilms demonstrates *Cryptosporidium*'s free-living potential and capacity for environmental survival and persistence, a characteristic of gregarines (Clode et al., 2015). For parasites like *Cryptosporidium* that use a faecal-oral transmission route with significant environmental exposure, the environmental persistence and infectivity of the oocyst and potentially other developmental stages will have a direct impact on local infection dynamics, including the ability to withstand extended periods without readily available hosts.

8. Significance of being a gregarine

We should not be surprised by the diversity so far demonstrated in the genus *Cryptosporidium*. Gregarines are ubiquitous, incredibly diverse parasites, in terms of species so far described, host range, and heterogeneity of life cycle patterns and developmental forms. The recognition of *Cryptosporidium*'s affinities with this group of protozoa helps to explain the increasing

Table 1
From coccidian to gregarine — influential observations.

Discovery	Date	Author
Cryptosporidium described from the stomach of a mouse with atypical coccidian features	1907	Tyzzer
Serological cross-reactivity of *Cryptosporidium* with a *Monocystis* sp. gregarine	1998	Bull et al.
18S sequencing of *Cryptosporidium* demonstrates a closer phylogenetic relationship to gregarine protozoa than to coccidians	1999	Carreno et al.
Novel stages described in the life cycle of *Cryptosporidium in vitro*	2002	Hijjawi et al.
Extracellular developmental stages of *Cryptosporidium* described *in vitro*	2004	Hijjawi et al.
Occurrence of syzygy described in *Cryptosporidium in vitro*	2004/2005	Hijjawi et al., and Rosales et al.
Gregarine nature of *Cryptosporidium*'s host parasite relationship demonstrated *in vivo*	2007	Valligurova et al.
Cryptosporidum capable of excystation and development in biofilms; occurrence of extracellular gamont stages confirmed	2013	Koh et al.
Molecular phylogenetic analysis supports transfer of *Cryptosporidium* from the coccidia to the class gregarinomorphea, as a new order and subclass of Gregarines	2014	Cavalier-Smith

Table 2
Biological and morphological similarities between *Cryptosporidium* and gregarines.*

Characteristic	Gregarines	*Cryptosporidium*
Apicoplast	Absent in some gregarines	Absent
Interface with host	Cell surface including epicellular and extracellular	Epicellular and extracellular
Multiplication	Multiple fission, merogony, gametogony, sporogony, binary fission, syzygy	Multiple fission, merogony, gametogony, sporogony, binary fission, syzygy
Morphology of developmental stages	Pleiomorphic, dependent on surrounding environment	Pleiomorphic, dependent on surrounding environment
Feeder organelle	Epimerite	Epimerite

* Details in Clode et al., 2015.

numbers of novel genotypes that are being discovered, and its expanding host range. It also emphasises that the specificity of environmental detection procedures for *Cryptosporidium* could be compromised by cross-reactivity with gregarine protozoa that are ubiquitous in fresh water environments (Bull et al., 1998; Hijjawi et al., 2002; Tenter et al., 2002). Recent studies have demonstrated the developmental heterogeneity of stages in the life cycle of *Cryptosporidium*, including extracellular trophozoites and gamonts (Borowski et al., 2008, 2010; Koh et al., 2013, 2014), that can survive in the environment, thus complicating surveillance and detection, especially if they are antigenically different to oocysts. The growing diversity will prove valuable fodder for taxonomists although the population structure of *Cryptosporidium* is uncertain and requires further study (Tibayrenc and Ayala, 2014). If we know the basic population structure of *Cryptosporidium*, predictions can be made on the extent to which genes are exchanged among genomes in the same population thus providing some stability to the species taxonomy of *Cryptosporidium*. In this respect, recent advances in nucleic acid-based approaches for the diagnosis and analysis of genetic diversity in species of *Cryptosporidium* (Jex et al., 2008) represent a significant step towards an improved understanding of the epidemiology and population structure (Beck et al., 2009).

We should also not be constrained by pre-conceived ideas in terms of drug discovery. It has been known for several years that *Cryptosporidium* has unique features that characterise its biochemistry and metabolism (Abrahamsen et al., 2004; Thompson et al., 2005; Zhu et al., 2000). Whether these are similar to those of other gregarines remains to be resolved, but the simplification of *Cryptosporidium*'s biosynthetic pathways (Clode et al., 2015) may enhance its ability to salvage nutrients from the environment. However, it opens the door to the development of relatively simple model drug screening systems utilising gregarines that can be readily maintained in their invertebrate hosts in the laboratory.

A better understanding of the developmental biology of *Cryptosporidium* in its host can now be achieved by a more comparative approach with what is known of some higher gregarines. This particularly applies to the parasite's relationship with its host cell and whether *Cryptosporidium*'s epimerite-like feeder organelle obtains nutrients in a way that is truly analogous to myzozytosis, as utilised by many gregarines, through which host cell contents are obtained. In addition, it is unclear how *Cryptosporidium*, which lacks key *de novo* synthesis pathways, acquires nutrients directly from an extracellular environment including the intestinal lumen of its host or the matrix of a biofilm. In this respect, it is interesting that the feeder organelle has been observed in extracellular stages in a biofilm environment (Koh et al., 2014) and thus may be able to acquire nutrients in such a host cell-free environment. Future studies not only will provide clues about the evolution of intracellular parasitism, but also will provide a better understanding of the host parasite relationship, as well as the development of the parasite in the environment.

Certainly, knowing that *Cryptosporidium* is a gregarine helps to explain why some key observations were at first difficult for some researchers to accept. However, this new understanding may help to advance research on *Cryptosporidium* and cryptosporidiosis in the future.

References

Abrahamsen, M.S., Templeton, T.J., Enomoto, S., Abrahante, J.E., Zhu, G., Lancto, C.A., Deng, M., Liu, C., Widmer, G., Tzipori, S., Buck, G.A., Xu, P., Bankier, A.T., Dear, P.H., Konfortov, B.A., Spriggs, H.F., Iyer, L., Anantharaman, V., Aravind, L., Kapur, V., 2004. Complete genome sequence of the apicomplexan, *Cryptosporidium parvum*. Science 304, 441–445.

Aldeyarbi, H.M., Karanis, P., 2016. The ultra-structural similarities between *Cryptosporidium parvum* and the gregarines. J. Eukaryot. Microbiol. 63, 79–85.

Angles, M.L., Chandy, J.P., Cox, P.T., Fisher, I.H., Warnecke, M.R., 2007. Implications of biofilm-associated waterborne *Cryptosporidium oocysts* for the water industry. Trends Parasitol. 23, 352–356.

Appelbee, A.J., Thompson, R.C.A., Olson, M.E., 2005. *Giardia* and *Cryptosporidium* in mammalian wildlife—current status and future needs. Trends Parasitol. 21, 370–376.

Barta, J.R., Thompson, R.C.A., 2006. What is *Cryptosporidium*? Reappraising its biology and phylogenetic affinities. Trends Parasitol. 22, 463–468.

Beck, H.P., Blake, D., Darde, M.L., Felger, I., Pedraza-Diaz, S., Regidor-Cerrillo, J., 2009. Molecular approaches to diversity of populations of apicomplexan parasites. Int. J. Parasitol. 39, 175–189.

Borowski, H., Clode, P.L., Thompson, R.C.A., 2008. Active invasion and/or encapsulation? A reappraisal of host-cell parasitism by Cryptosporidium. Trends Parasitol. 24, 509–516.

Borowski, H., Thompson, R.C.A., Armstrong, T., Clode, P.L., 2010. Morphological characterization of *Cryptosporidium parvum* life-cycle stages in an in vitro model system. Parasitology 137, 13–26.

Bull, S., Chalmers, R., Sturdee, A.P., A., C., Kennaugh, J., 1998. Cross-reaction of an anti-*Cryptosporidium* monoclonal antibody with sporocysts of *Monocystis* species. Vet Parasitol. 77, 195–197.

Cacciò, S.M., Thompson, R.C., McLauchlin, J., Smith, H.V., 2005. Unravelling *Cryptosporidium* and *Giardia* epidemiology. Trends Parasitol. 21, 430–437.

Carreno, R.A., Martin, D.S., Barta, J.R., 1999. Cryptosporidium is more closely related to the gregarines than to coccidia as shown by phylogenetic analysis of apicomplexan parasites inferred using small-subunit ribosomal RNA gene sequences. Parasitol. Res. 85, 899–904.

Casemore, D.P., Wright, J.T., Coop, R.L., 1997. Cryptosporidiosis-human and animal epidemiology. In: Fayer, R. (Ed.), *Cryptosporidium* and *Cryptosporidiosis*. CRC Press, Florida, pp. 65–92.

Cavalier-Smith, T., 2014. Gregarine site-heterogeneous 18S rDNA trees, revision of gregarine higher classification, and the evolutionary diversification of sporozoa. Eur. J. Protistol. 50, 472–495.
Checkley, W., White, C., Jaganath, D., Arrowood, M.J., Chalmers, R.M., Chen, X.-M., et al., 2014. A review of the global burden, novel diagnostics, therapeutics and vaccine targets for cryptosporidiosis. Lancet Inf. Dis. 15, 85–94.
Clode, P., Koh, W., Thompson, R.C.A., 2015. Life without a host cell: what is *Cryptosporidium*? Trends Parasitol. 614–624.
Elliot, A., Morgan, U.M., Thompson, R.C.A., 1999. Improved staining method for detecting *Cryptosporidium* oocysts in stools using Malachite Green. J. Gen. Appl. Microbiol. 45, 139–142.
FAO, 2014. Multicriteria-Based Ranking for Risk Management of Food-Borne Parasites. Microbiological Risk Assessment Series 23. Food Safety and Codex Unit, Food and Agriculture Organization of the United Nations, Rome, Italy.
Fayer, R., Morgan, U., Upton, S.J., 2000. Epidemiology of *Cryptosporidium:* transmission, detection and identification. Int. J. Parasitol. 30, 1305–1322.
Fletcher, S.M., Stark, D., Harkness, J., Ellis, J., 2012. Enteric protozoa in the developed world: a public health perspective. Clin. Microbiol. Rev. 25, 420–449.
Gajadhar, A.A., Lalonde, L.F., Al-Adhami, B., Singh, B.B., Lobanov, V., 2015. Foodborne apicomplexan protozoa: coccidia. In: Gajadhar, A. (Ed.), Foodborne Parasites: in The Food Supply Web. Elsevier, pp. 101–147.
Hijjawi, N.S., Meloni, B.P., Morgan, 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.M., 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.
Hotez, P.J., Bottazzi1, M.E., Strych, U., Chang, L.-Y., Lim, Y.A.L., Goodenow, M.M., AbuBakar, S., 2015. Neglected tropical diseases among the Association of Southeast Asian Nations (ASEAN): overview and update. PLoS Negl. Trop. Dis 9 (4), e0003575.
Howe, A.D., Forster, S., Morton, S., Marshall, R., Osborn, K.S., Wright, P., Hunter, P.R., 2002. *Cryptosporidium* oocysts in a water supply associated with a cryptosporidiosis outbreak. Emerg. Infect. Dis. 8, 619–624.
Hunter, P.R., Thompson, R.C.A., 2005. The zoonotic transmission of *Giardia* and *Cryptosporidium*. Int. J. Parasitol. 35, 1181–1190.
Jex, A.R., Smith, H.V., Monis, P.T., Campbell, B.E., Gasser, R.B., 2008. *Cryptosporidium* – biotechnological advances in the detection, diagnosis and analysis of genetic variation. Biotech. Adv. 26, 304–317.
Karanis, P., Aldeyarbi, H.M., 2011. Evolution of *Cryptosporidium in vitro* culture. Int. J. Parasitol. 41, 1231–1242.
Karanis, P., Kourenti, C., Smith, H., 2007. Waterborne transmission of protozoan parasites: a worldwide review of outbreaks and lessons learnt. J. Water Health 5, 1–38.
Karanis, P., Kimura, A., Nagasawa, H., Igarashi, I., Suzuki, N., 2008. Observations on *Cryptosporidium* life cycle stages during excystation. J. Parasitol. 94, 298–300.
Koh, W., Clode, P.l., Monis, P., Thompson, R.C.A., 2013. Multiplication of the waterborne pathogen *Cryptosporidium parvum* in an aquatic biofilm system. Parasit. Vectors 6, 270.
Koh, W., Thompson, R.C.A., Edwards, H., Monis, P., Clode, P.L., 2014. Extracellular excystation and development of *Cryptopsoridium*: 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., et al., 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., Ramey, P.A., 2006. Cellular identity of a novel small subunit rDNA sequence clade of apicomplexans: description of the marine parasite *Rhytidocystis polygordiae* n.sp. (host: *Poly-gordius* sp., Polychaeta). J. Eukartot. Microbiol. 53, 280–291.
Levine, N.D., 1988. The Protozoan Phylum Apicomplexa (Vols 1 and 2). CRC Press.
Lymbery, A.J., Thompson, R.C.A., 2011. The molecular epidemiology of parasite infections: tools and applications. Mol. Biochem. Parasitol. 181, 102–116.
Mbae, C.K., Nokes, D.J., Mulinge, E., Nyambura, J., Waruru, A., Kariuki, S., 2013. Intestinal parasitic infections in children presenting with diarrhoea in outpatient and inpatient settings in an informal settlement of Nairobi, Kenya. BMC Infect. Dis. 13, 243.
Millard, P.S., Gensheimer, K.F., Addiss, D.G., Sosin, D.M., Beckett, G.A., Houck-Jankoski, A., Hudson, A., 1994. An outbreak of cryptosporidiosis from fresh-pressed apple cider. JAMA 272, 1592–1596.
Nyarango, R.M., Aloo, P.A., Kabiru, E.W., Nyanchongi, B.O., 2008. The risk of pathogenic intestinal parasite infections in Kisii Municipality, Kenya. BMC Pub. Health 8, 237.
O'Donoghue, P.J., 1995. *Cryptosporidium* and *Cryptosporidiosis* in man and animals. Int. J. Parasitol. 85, 525–530.
Oates, S.C., Miller, M.A., Hardin, D., Conrad, P.A., Melli, A., Jessup, D.A., Dominik, C., Roug, A., Tinker, M.T., Miller, W.A., 2012. Prevalence, environmental loading, and molecular characterization of *Cryptosporidium* and *Giardia* isolates from domestic and wild animals along the Central California Coast. Appl. Environ. Microbiol. 78, 8762–8772.
Ortega, Y.R., Sheehy, R.R., Cama, V.A., Oishi, K.K., Sterling, C.R., 1991. Restriction fragment length polymorphism analysis of *Cryptosporidium parvum* isolates of bovine and human origin. J. Protozool. 38, 40–41.
Peng, M.M., Xiao, L., Freeman, A.R., Arrowood, M.J., Escalante, A.A., Weltman, A.C., et al., 1997. Genetic polymorphism among *Cryptosporidium parvum* isolates: evidence of two distinct human transmission cycles. Emer. Inf. Dis. 34, 567–573.
Rosales, M.J., Cordón, G.P., Moreno, M.S., Sánchez, C.M., Mascaró, C., 2005. Extracellular like-gregarine stages of *Cryptosporidium parvum*. Acta Trop. 95, 74–78.
Rose, J.B., Slifko, T.R., 1999. *Giardia*, *Cryptosporidium*, and *Cyclospora* and their impact on foods: a review. J. Food. Prot. 62, 1059–1070.
Savioli, L., Smith, H., Thompson, R.C.A., 2006. *Giardia* and *Cryptosporidium* join the 'Neglected Diseases Initiative'. Trends Parasitol. 22, 203–208.
Searcy, K.E., Packman, A.I., Atwill, E.R., Harter, T., 2006. Capture and retention of *Cryptosporidium parvum* oocysts by *Pseudomonas aeruginosa* biofilms. Appl. Environ. Microbiol. 72, 6242–6247.
Sibley, L.D., 2004. Intracellular parasite invasion strategies. Science 304, 248–253.
Slapeta, J., 2011. Naming of *Cryptosporidium pestis* is in accordance with the ICZN Code and the name is available for this taxon previously recognized as *C. parvum* 'bovine genotype'. Vet. Parasitol. 177, 1–5.
Slapeta, J., 2013. Cryptosporidiosis and *Cryptosporidium* species in animals and humans: a thirty colour rainbow? Int. J. Parasitol. 43, 957–970.
Smith, H.V., Caccio, S.M., Tait, A., McLauchlin, J., Thompsoin, R.C.A., 2006. Tools for investigating the environmental transmission of *Cryptosporidium* and *Giardia* infections in humans. Trends Parasitol. 22, 160–167.
Smith, H.V., Cacciò, S.M., Cook, N., Nichols, R.A., Tait, A., 2007. *Cryptosporidium* and *Giardia* as foodborne zoonoses. Vet. Parasit. 149, 29–40.
Szostakowska, B., Kruminis-Lozowska, W., Racewicz, M., Knight, R., Tamang, L., Myjak., P., et al., 2004. *Cryptosporidium parvum* and *Giardia lamblia* recovered from flies on a cattle farm and in a landfill. App. Environ. Micro. 70, 3742–3744.
Tellevik, M.G., Moyo, S.J., Blomberg, B., Hjøllo, T., Maselle, S.Y., Langeland, N., et al., 2015. Prevalence of *Cryptosporidium parvum/hominis*, *Entamoeba histolytica* and *Giardia lamblia* among young children with and without diarrhea in Dar es Salaam, Tanzania. PLoS Negl. Trop Dis 9 (10), e0004125.
Tenter, A.M., Barta, J.R., Beveridge, I., Duszynski, D.W., Mehlhorn, H., Morrison, D.A., Thompson, R.C.A., Conrad, P.A., 2002. The conceptual basis for a new classification of the coccidia. Int. J. Parasitol. 32, 595–616.
Thompson, R.C.A., 2003. Molecular epidemiology of *Giardia* and *Cryptosporidium* infections. J. Parasitol. 89, S134–S140.
Thompson, R.C.A., Ash, A., 2015. Molecular epidemiology of *Giardia* and *Cryptosporidium* infections. Inf. Gen. Evol. 40, 315–323.
Thompson, R.C.A., Smith, A., 2011. Zoonotic enteric protozoa. Vet. Parasitol. 182, 70–78.
Thompson, R.C.A., Olson, M.E., Zhu, G., Enomoto, S., Abrahamsen, M.S., Hijjawi, N.S., 2005. *Cryptosporidium* and cryptosporidiosis. Adv. Parasitol. 59, 77–158.
Thompson, R.C.A., Traub, R.J., Parameswaran, N., Fried, B., 2007. Molecular epidemiology of foodborne parasitic zoonoses. In: Murrell, K.D. (Ed.), Food-Borne Parasitic Zoonoses. Spinger, pp. 383–415.
Tibayrenc, M., Ayala, F.J., 2014. *Cryptosporidium*, *Giardia*, *Pneumocystis* genetic variability: cryptic biological species or clonal near-clades? PLoS Pathog. 10 (4), e1003908.
Tumwine, J.K., Kekitiinwa, A., Bakeera-Kitaka, S., Ndeezi, G., Downing, R., Feng, X., et al., 2005. *Cryptosporidiosis* and microsporidiosis in Ugandan children with persistent diarrhea with and without concurrent infection with the human immunodeficiency virus. Am. J. Trop. Med. Hyg. 73, 921–925.
Tyzzer, E.E., 1907. A sporozoan found in the peptic glands of the common mouse. Proc. Soc. Exp. Biol. Med. 5, 12–13.

Valigurová, A., Hofmannová, L., Koudela, B., Vra, J., 2007. An ultrastructural comparison of the attachment sites between *Gregarina steini* and *Cryptosporidium muris*. J. Eukaryot. Microbiol. 54, 495–510.
Valigurová, A., Jirků, M., Koudela, B., Gelnar, M., Modrý, D., Šlapeta, J., 2008. Cryptosporidia: epicellular parasites embraced by the host cell membrane. Int. J. Parasitol. 38, 913–922.
Valigurová, A., Vaskovicová, N., Musilová, N., Schrével, J., 2013. The enigma of eugregarine epicytic folds: where gliding motility originates? Front. Zool. 10, 57.
Xiao, L., 2010. Molecular epidemiology of cryptosporidiosis: an update. Exp. Parasitol. 124, 80–89.
Xiao, L., Fayer, R., 2008. Molecular characterisation of species and genotypes of *Cryptosporidium* and *Giardia* and assessment of zoonotic transmission. Int. J. Parasitol. 38, 1239–1255.
Zhu, G., Marchewka, M.J., Keithly, J.S., 2000. *Cryptosporidium parvum* appears to lack a plastid genome. Microbiology 146, 315–321.