Cryptosporidium

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Contents

Abstract According to the World Health Organisation, cryptosporidiosis is a global diarrhoeal disease affecting millions of individuals; it is the second most common cause of infantile death in developing countries and is increasingly identified as an emerging cause of morbidity and mortality worldwide. The disease is also extremely

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severe in livestock, causing profuse diarrhoea and considerable economic losses in farmed young animals. Given the lack of effective treatment (absence of vaccines and effective drugs) and the limited understanding of the causative parasite, cryptosporidiosis represents a major challenge in the battle against global diarrhoeal diseases. Currently, there are 45 described *Cryptosporidium* species infecting a whole spectrum of animals. In this book chapter we will present an overview of the parasite, focusing on its taxonomic status, its morphology, its prevalence and transmission. We will review both cell biological and molecular techniques currently used to investigate the biology of this parasite and we will introduce the new state-of-the-art techniques that have been established by several laboratories in the field. With the development of these new technologies, we will be able to further understand the unique biology of *Cryptosporidium* and its role in health and disease of its host.

1 Introduction

Cryptosporidium is a genus of single-cell microbial parasites that infect the gut of a diverse range of vertebrate species causing mild to severe diarrhoea. In humans, several *Cryptosporidium* species have been shown to cause cryptosporidiosis (Nader et al. 2019), a global diarrhoeal disease affecting millions of individuals; it is the second most common cause of infantile death in developing countries and is increasingly identified as an emerging cause of morbidity and mortality worldwide (Kotloff et al. 2013; Checkley et al. 2015; Platts-Mills et al. 2015). The disease is also extremely severe in livestock, causing profuse diarrhoea and considerable economic losses in farmed young calves and lambs (Joachim et al. 2003; Thompson and Ash 2016). Given the lack of effective treatment (absence of vaccines and effective drugs) and the limited understanding of the causative parasite, cryptosporidiosis represents a major challenge in the battle against global diarrhoeal diseases.

In this book chapter we will present the status quo on *Cryptosporidium*: from its taxonomy, to its biology and the host-parasite interactions, both in the cellular, but also within a community level (e.g. gut microbiome).

2 *Cryptosporidium* Species and Host Specificity

Cryptosporidium spp. are parasites taxonomically assigned to the Apicomplexan phylum, which was until recently further divided into class Conoidasida, subclass Coccidia, order Eucoccidiida, suborder Eimeriorina and family Cryptosporidiidae, comprising only one genus, *Cryptosporidium* (Integrated Taxonomic Information System 2020). Recently, new genomic and phylogenetic studies (Liu et al. 2016; Ryan et al. 2016a) denoted that *Cryptosporidium* presents more similarities with

Gregarinia than with Coccidia subclass leading to an urge in reclassifying this microorganism with its own subclass Cryptogregarinorida (Adl et al. 2019).

With the advent of molecular biology detection methods, particularly the Polymerase Chain Reaction (PCR), a great progress was rendered regarding detection and differentiation of *Cryptosporidium* spp. occurring in both clinical and environmental settings (Smith et al. 2006; Thompson and Ash 2016). Among the PCR-based tools, targeting and amplification of the small subunit rRNA (18S rRNA) gene followed by sequencing, allowed to truly distinguish and characterize *Cryptosporidium* at the species level (Xiao et al. 1999, 2004; Xiao 2010; Xiao and Feng 2017) (Fig. 1). Due to the progress of the aforementioned molecular diagnostic techniques a total of 45 species have been documented so far (Table 1). Among these recognized species, there are significant differences, not only morphologically (Table 2), but also on the site of infection in the respective host: the majority of them infecting the intestine, but some have been found to invade the stomach instead. Interestingly, multiple infections in different parts of the host are not uncommon, and on occasion *Cryptosporidium* species have been identified in the respiratory tract of the host as well (Sponseller et al. 2014).

2.1 Cryptosporidium Infection and Zoonotic Transmission

Although *Cryptosporidium* was described for the first time in 1907 (Tyzzer 1907), it took more than 50 years to be reported for the first time in humans (Meisel et al. 1976; Nime et al. 1976). Since then, more than 20 species/genotypes have been associated with human infections (Feng et al. 2018; Santos et al. 2019). The majority of these human pathogenic *Cryptosporidium* species/genotypes display host promiscuity, meaning that they can be found and cause disease in several different host species and affect both immunocompetent and immunocompromised individuals (Ryan et al. 2016b; Xiao and Feng 2017; Feng et al. 2018). Furthermore, it is speculated that more *Cryptosporidium* species/genotypes found in mammals could be linked with human infections as well (Xiao and Feng 2017). Therefore, it is of utmost importance to characterize *Cryptosporidium* spp. at species/genotype level to determine their human infectivity, incidence and public health hazard (Morris et al. 2019).

Cryptosporidium transmission routes are usually split into two categories: direct and indirect (Cacciò and Chalmers 2016). The direct pathway involves contact with an infected host and transmission occurs through the faecal-oral route (person-to-person, animal-to-person or person-to-animal), whilst the indirect pathway encompasses infections through interaction and inadvertent intake of material containing *Cryptosporidium*, such as water (via drinking or recreational activities), soil, fomites and food (Cacciò and Chalmers 2016; Innes et al. 2020). More recently, it was found that inhalation of *Cryptosporidium* oocysts embedded in droplets might be an overlooked source of infection, particularly in immunocompromised patients (Sponseller et al. 2014; Abdoli et al. 2018; Nyangulu et al. 2019; Xiao and Griffiths 2020).

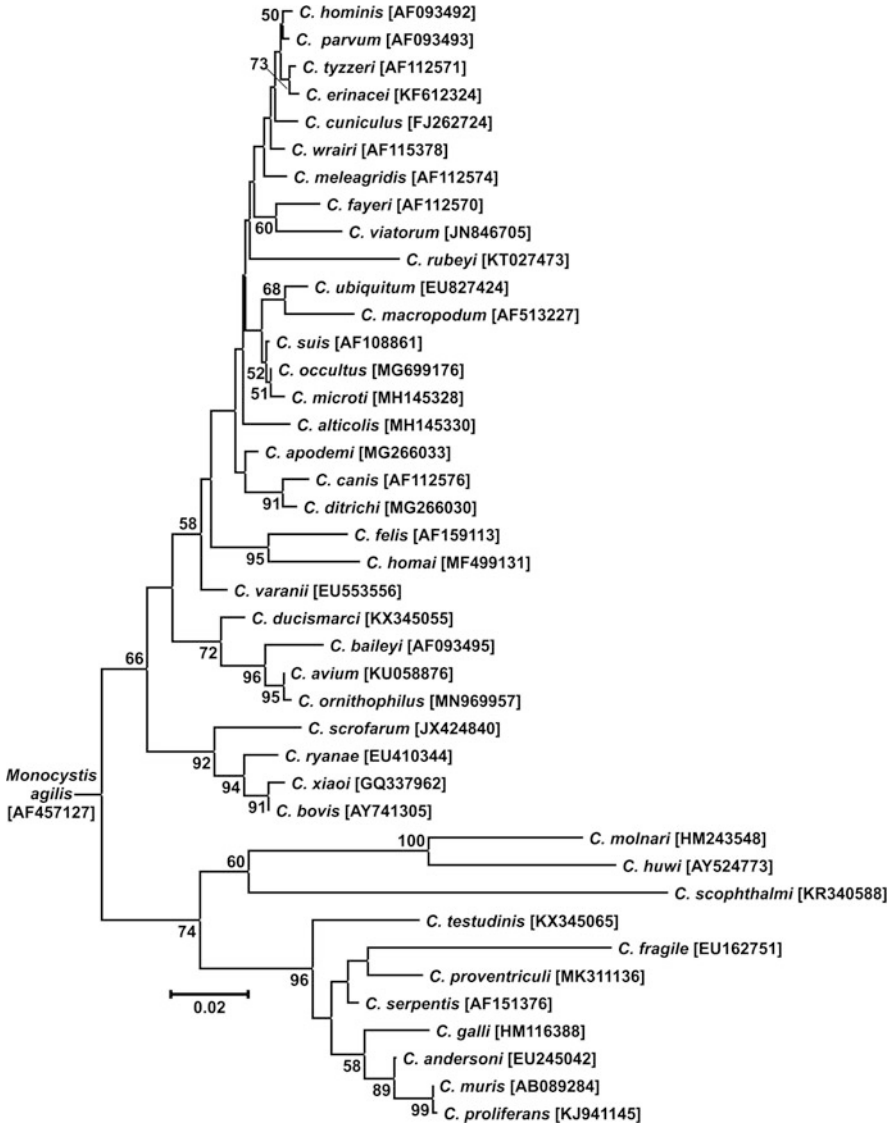

Fig. 1 Neighbour-joining tree based on partial sequences of the gene encoding the small subunit rRNA valid *Cryptosporidium* species. Tamura's 3-parameter model was applied. The robustness of the phylogeny was tested with 1000 bootstrap and numbers at the nodes represent the bootstrap *p*-values with more than 50% bootstrap support. The branch length scale bar, indicating the number of substitutions per site is included. The GenBank Accession number is in parenthesis

Table 1 Currently accepted *Cryptosporidium* species, main hosts, infection site and human pathogenicity

Species	Main host (s)	Site of infection	Associated with human infections[a]	Subtype families based on *gp60* sequences	References
C. alticolis	Common voles	Small intestine	No	–	Horčičková et al. (2019)
C. andersoni	Cattle	Stomach	Yes (minor)	–	Lindsay et al. (2000)
C. apodemi	Field mice	Intestine	No	–	Čondlová et al. (2018)
C. avium	Birds	Intestinal and urinary tract	No	–	Holubová et al. (2016)
C. baileyi	Poultry	Respiratory, urinary and intestinal tract	Yes (occasional)	–	Current et al. (1986)
C. bovis	Cattle	Small intestine	Yes (occasional)	–	Fayer et al. (2005)
C. canis	Dogs	Small intestine	Yes (minor)	–	Fayer et al. (2001)
C. cuniculus	European rabbits and humans	Small intestine	Yes (minor)	Va, Vb	Inman and Takeuchi (1979), Robinson et al. (2010)
C. ditrichi	Field mice	Small intestine	Yes (occasional)	–	Čondlová et al. (2018)
C. ducismarci	Tortoises	Intestine	No	–	Traversa (2010), Ježková et al. (2016)
C. erinacei	Hedgehogs	Small intestine	Yes (occasional)	XIIIa	Kváč et al. (2014a)
C. fayeri	Marsupials	Small intestine	Yes (occasional)	IVa, IVb, IVc, IVd, IVe, IVf	Ryan et al. (2008)
C. felis	Cats	Small intestine	Yes (occasional)	XIX	Iseki (1979)
C. fragile	Black spined toads	Stomach	No	–	Jirků et al. (2008)
C. galli	Birds	Proventriculus	No	–	Ryan et al. (2003b)
C. homai	Guinea pigs	Intestine	No	–	Zahedi et al. (2017)

(continued)

Table 1 (continued)

Species	Main host (s)	Site of infection	Associated with human infections[a]	Subtype families based on *gp60* sequences	References
C. hominis	Humans	Small intestine	Yes (major)	Ia, Ib, Id, Ie, If, Ig, Ih, Ii, Ij, Ik	Morgan-Ryan et al. (2002)
C. huwi	Guppy	Stomach	No	–	Ryan et al. (2015)
C. macropodum	Marsupials	Intestine	No	–	Power and Ryan (2008)
C. meleagridis	Poultry and humans	Intestine	Yes (minor)	IIIa, IIIb, IIIc, IIId, IIIe, IIIf, IIIg, IIIh, IIIi, IIIj	Slavin (1955)
C. microti	Common vole	Large intestine	No	–	Horčičková et al. (2019)
C. molnari	Sea bream and sea bass	Intestine	No	–	Alvarez-Pellitero and Sitjà-Bobadilla (2002)
C. muris	Rodents	Stomach	Yes (minor)	–	Tyzzer (1907, 1910)
C. occultus	Rats	Large intestine	Yes (Occasional)	–	Kváč et al. (2018)
C. parvum	Ruminants (mainly pre—weaned) and humans	Small intestine	Yes (major)	IIa, IIb, IIc, IId, IIe, IIg, IIh, IIi, IIk, III, IIm, IIn, IIo, IIp, IIq, IIr, IIs, IIt	Upton and Current (1985)
C. proliferans	Rodents and possibly equids	Stomach	No	–	Kváč et al. (2016)
C. proventriculi	Parrots	Proventriculus and ventriculus	No	–	Holubová et al. (2019)
C. rubeyi	Ground squirrels	Unknown	No	–	Li et al. (2015b)
C. ryanae	Cattle	Small intestine	No	–	Fayer et al. (2008)
C. scrofarum	Pigs	Intestine	Yes (occasional)	–	Kváč et al. (2013)
C. serpentis	Snakes and lizards	Stomach	No	–	Levine (1980)

(continued)

Table 1 (continued)

Species	Main host (s)	Site of infection	Associated with human infections[a]	Subtype families based on *gp60* sequences	References
C. suis	Pigs	Intestine	Yes (occasional)	–	Ryan et al. (2004)
C. testudinis	Tortoises	Unknown	No	–	Ježková et al. (2016)
C. tyzzeri	Rodents	Small intestine	Yes (occasional)	IXa, IXb	Ren et al. 2012)
C. ubiquitum	Several mammals	Intestine	Yes (minor)	XIIa, XIIb, XIIc, XIId, XIIg, XIIh	Fayer et al. (2010)
C. varanii	Lizards and snakes	Intestine	No	–	Pavlásek and Ryan (2008)
C. viatorum	Humans	Unkown	Yes (minor)	XVa, XVb, XVc, XVd	Elwin et al. (2012b)
C. wrairi	Guinea pigs	Small intestine	Yes (occasional)	VIIa	Vetterling et al. (1971)
C. xiaoi	Sheep and goats	Unknown	Yes (occasional)	–	Fayer and Santín (2009)

Adapted from Ryan et al. (2014), Xiao and Feng (2017), Feng et al. (2018), Morris et al. (2019)
[a]Major (responsible for more than 90% of infections in humans), minor (responsible for more than five cases in humans), occasional (responsible for less than five cases in humans)

2.1.1 Genotyping *Cryptosporidium parvum* and *C. hominis*

The most predominant *Cryptosporidium* species to infect humans are *C. parvum* and *C. hominis*, which account for more than 90% of cryptosporidiosis manifestations observed (Xiao and Feng 2008; Bouzid et al. 2013). These species used to be deemed as two different subtypes of *C. parvum* until 2002, when analysis using new molecular markers combined with distinct host tropisms lead to establishing *C. hominis* as a distinct species (Morgan-Ryan et al. 2002; Kissinger 2019; Nader et al. 2019). With the advance of *Cryptosporidium* molecular epidemiology and PCR-based subtyping tools, mainly targeting the gene that encodes for the 60-kDa glycoprotein (*gp60*), it was possible to uncover several differences between these two species in terms of transmission routes, geographic distribution, socio-economic backgrounds, temporal and age-associated variations (Xiao 2010; Ryan et al. 2014; Xiao and Feng 2017; Feng et al. 2018; Nader et al. 2019; Santos et al. 2019).

The *gp60* (also labelled as *gp40/15*) gene encodes a precursor protein, further cleaved to generate mature cell surface glycoproteins (gp40 and gp15) involved in binding and invasion of enterocytes (Xiao 2010; Ryan et al. 2014; Xiao and Feng

Table 2 Oocyst size of valid *Cryptosporidium* species

Species/genotype	Oocyst size (μm)	Length/ width ratio	References
C. alticolis	4.9–5.7 × 4.6–5.2	1.00–1.20	Horčičková et al. (2019)
C. andersoni	6.0–8.1 × 5.0–6.5	1.07–1.50	Lindsay et al. (2000)
C. apodemi	3.9–4.7 × 3.8–4.4	1.00–1.06	Čondlová et al. (2018)
C. avium	5.30–6.90 × 4.30–5.50	1.14–1.47	Holubová et al. (2016)
C. baileyi	6.0–7.5 × 4.8–5.7	1.05–1.79	Current et al. (1986), Lindsay et al. (1989)
C. bovis	4.76–5.35 × 4.17–4.76	1.06	Fayer et al. (2005)
C. canis	3.68–5.88 × 3.68–5.88	1.04–1.06	Fayer et al. (2001)
C. cichlidis	4.0–4.7 × 2.5–3.5	–	Paperna and Vilenkin (1996)
C. cuniculus	5.55–6.40 × 5.02–5.92	1.11	Robinson et al. (2010)
C. ditrichi	4.5–5.2 × 4.0–4.6	1.0–1.2	Čondlová et al. (2018)
C. ducismarci	4.4–5.4 × 4.3–5.3	1.1 ± 0.03	Traversa (2010), Ježková et al. (2016)
C. erinacei	4.5–5.8 × 4.0–4.8	1.02–1.35	Kváč et al. (2014a)
C. fayeri	4.5–5.1 × 3.8–5.0	1.02–1.18	Ryan et al. (2008)
C. felis	5.0 × 4.5	–	Iseki (1979)
C. fragile	5.5–7.0 × 5.0–6.5	1.0–1.3	Jirků et al. (2008)
C. galli	8.0–8.5 × 6.2–6.4	1.3	Ryan et al. (2003b)
C. homai	Unknown	–	Zahedi et al. (2017)
C. hominis	4.4–5.4 × 4.4–5.9	*1.01–1.*09	Morgan-Ryan et al. (2002)
C. huwi	4.4–4.9 × 4.0–4.8	0.92–1.35	Ryan et al. (2015)
C. macropodum	4.5–6.0 × 5.0–6.0	1.1	Power and Ryan (2008)
C. meleagridis	4.5–6.0 x 4.2–5.3	1.00–1.33	Slavin (1955), Lindsay et al. (1989)
C. microti	3.9–4.7 × 3.8–4.4	1.00–1.06	Horčičková et al. (2019)
C. molnari	3.23–5.45 × 3.02–5.04	1.00–1.17	Sitjà-Bobadilla et al. (2006)
C. muris	6.6–7.9 × 5.3–6.5	1.1–1.5	Tyzzer (1910), Upton and Current (1985)
C. nasoris	3.6	–	Hoover et al. (1981)
C. occultus	4.66–5.53 × 4.47–5.44	1.00–1.17	Kváč et al. (2018)
C. ornithophilus	5.24–6.77 × 4.68–5.50	1.06–1.36	Holubová et al. (2020)
C. parvum	4.5–5.4 × 4.5–5.4	1.0–1.3	Tyzzer (1912), Upton and Current (1985)
C. proliferans	6.8–8.8 × 4.8–6.2	1.48	Kváč et al. (2016)
C. proventriculi	6.70–8.40 × 5.10–6.3	1.08–1.41	Holubová et al. (2019)
C. reichenbachklinkei	2.4–3.18 × 2.4–3.0	–	Paperna and Vilenkin (1996)
C. rubeyi	4.4–5.0 × 4.0–5.0	1.08	Li et al. (2015b)
C. ryanae	2.94–4.41 × 2.94–3.68	1.18	Fayer et al. (2008)
C. scophthalmi	3.7–5.03 × 3.03–4.69	1.05–1.34	Alvarez-Pellitero et al. (2004)

(continued)

Table 2 (continued)

Species/genotype	Oocyst size (μm)	Length/ width ratio	References
C. scrofarum	4.81–5.96 × 4.23–5.29	1.07 ± 0.06	Kváč et al. (2013)
C. serpentis	6.3 × 5.5	1.14 ± 0.11	Levine (1980), Graczyk et al. (1995)
C. suis	6.0–6.8 × 5.3–5.7	1.14	Ryan et al. (2004), Vítovec et al. (2006)
C. testudinis	5.8–6.9 × 5.3–6.5	1.1 ± 0.05	Ježková et al. (2016)
C. tyzzeri	4.64 ± 0.05 × 4.19 ± 0.06	1.11 ± 0.06	Ren et al. (2012)
C. ubiquitum	4.71–5.32 × 4.33–4.98	1.08	Fayer et al. (2010)
C. varanii	4.8–5.1 × 4.4–4.8	1.03 ± 0.03	Pavlásek et al. (1995)
C. viatorum	4.87–5.87 × 4.15–5.20	1.03–1.32	Elwin et al. (2012b)
C. wrairi	4.0–5.0 × 4.8–5.6	–	Vetterling et al. (1971)
C. xiaoi	2.94–4.41 × 2.94–4.41	1.15	Fayer and Santín (2009)

2017). At the 5′-end of the *gp60* gene there is a region analogous to a microsatellite sequence which contains tandem repeats of the serine-coding trinucleotide TCA, TCG or TCT (Xiao 2010; Ryan et al. 2014; Xiao and Feng 2017). These trinucleotide repeats vary in numbers, which are then employed to further characterize the subtype families within a species (Xiao and Feng 2017; Chalmers et al. 2019). The remaining gene sequence consists of a conserved region that identifies the allelic family and, immediately downstream of the trinucleotides repeats, copies of repetitive sequences which help discriminate the subtype even further (Ryan et al. 2014; Chalmers et al. 2019).

2.1.2 *Cryptosporidium parvum*

Cryptosporidium parvum is presently the most important zoonotic species and displays a wide number of mammal hosts, with particular incidence in humans and young livestock (Table 1), leading to a growing concern that the parasite might be adjusting and thriving under several hosts (Šlapeta 2013; Zahedi et al. 2016b; Feng et al. 2018; Pumipuntu and Piratae 2018). Up to date, almost 20 different subtypes families were described (IIa to IIt), with IIa, IIc, and IId subtypes being regarded as the most common subtypes observed in human infections (Xiao and Feng 2017; King et al. 2019).

The subtype IIc is a human-adapted *C. parvum* anthroponotic subtype (spread between humans with no animal vector), which is the major source of *C. parvum* infections in low-income countries with poor sanitation conditions as well as in HIV-positive individuals (King et al. 2019). Until very recently, only three studies established the *C. parvum* IIc subtype outside of human hosts, more concretely in hedgehogs (Dyachenko et al. 2010; Krawczyk et al. 2015; Sangster et al. 2016). However, a study released in 2019 found the *C. parvum* subtype IIc in rabbits from

Nigeria, which might question the anthroponotic status of the IIc subtype and definitely warrants further studies on the zoonotic potential of this subtype (Ayinmode and Agbajelola 2019).

In contrast, the subtype IIa, which is a well-documented zoonotic *C. parvum* family, tends to dominate in richer nations (Xiao 2010; Ryan et al. 2014; Feng et al. 2018; Innes et al. 2020). The subtype IIa, which is exceptionally prevalent in pre-weaned calves, is linked with various occurrences of zoonotic transmission (both sporadic and outbreak cases) of *C. parvum* in Europe involving animals and humans (Stensvold et al. 2015b; Cacciò and Chalmers 2016; Chalmers et al. 2019). In North America, New Zealand and Australia a similar outcome was also observed, with most *C. parvum* infections being associated with subtype IIa (King et al. 2019). In fact, cattle has been tied with cryptosporidiosis manifestations in humans since the 1980s, with initial reports pointing the likelihood of *Cryptosporidium* infections after contact with infected calves (Fayer et al. 2000; Preiser et al. 2003; Smith et al. 2004; Kiang et al. 2006; Xiao and Feng 2008). Further research also pointed towards an increase exposure to cryptosporidiosis whilst living in rural environments due to *C. parvum*, since human contact with domestic animals reservoirs of the parasite are more common and activities involving livestock by-products, such as manure land applying, are also more common (Lake et al. 2007; Pollock et al. 2010).

The subtype IId is primarily found in sheep/goats, and it is mostly associated with zoonotic transmission and infection of *Cryptosporidium* to humans in the Middle east region (Ryan et al. 2014; Xiao and Feng 2017; King et al. 2019). Notably, the IId family occurrence in China appears to be highly prevalent in pre-weaned calves contrasting with the prevalence of the IIa type in pre-weaned calves from other parts of the world (Feng and Xiao 2017). In addition, numerous studies indicated cross-species transmission of IId subtypes among goats, horses, donkeys, and takins whilst further research also detected this *Cryptosporidium* subtype in humans living in China, drawing attention to the likely zoonotic transmission potential of this subtype family (Feng and Xiao 2017).

Lastly, although *C. parvum* is largely associated with transmission and infection in mammals, recent studies highlighted its presence in edible fish (sea and freshwater) (Roberts et al. 2007; McOliver et al. 2009; Reid et al. 2010; Koinari et al. 2013; Certad et al. 2015, 2019; Couso-Pérez et al. 2018), raising awareness for a new possible non-mammal zoonotic transmission route of *C. parvum* concerning humans, as well as the impact of human and cattle waste discharge into the ocean, since most of the fish were affected by the *C. parvum* subtype IIa (Reid et al. 2010; Koinari et al. 2013; Certad et al. 2015, 2019).

2.1.3 *Cryptosporidium hominis*

Cryptosporidium hominis, is a species considered to be almost exclusive in humans and thus anthroponotic, sharing a common transmission route with *C. parvum* IIc subtype (Kissinger 2019; Nader et al. 2019). It is described as responsible for most of the cryptosporidiosis cases observed in humans in developing countries and as

evenly responsible for most of the human infections with *C. parvum* in developed countries (Xiao 2010; Ryan et al. 2014). It comprises less subtype families than *C. parvum* with only 10 subtype families (Ia to Ik) (Xiao and Feng 2017). The most prevalent subtype family in human infections belongs to the Ib family, which is broadly spread around the globe in both resource-rich and resource-poor countries (Feng et al. 2018).

Until recently, it was thought that *C. hominis* was mostly restricted to humans. However, an increasing number of researchers are finding evidence of *C. hominis* in both wild and domestic animals (Widmer et al. 2020). Examples include non-human primates, livestock, marsupials, rodents, hedgehogs, carnivores, bats, marine mammals, birds and fish (Morgan et al. 2000; Zhou et al. 2004; Koinari et al. 2013; Krawczyk et al. 2015; Laatamna et al. 2015, 2015; Schiller et al. 2016; Danišová et al. 2017; Mateo et al. 2017; Zahedi et al. 2018; Chen et al. 2019a; Hatam-Nahavandi et al. 2019; Zhao et al. 2019b; Widmer et al. 2020). Considering that infections with subtype Ib were also regularly found among some of these animals, a reverse zoonotic spread from humans to animals appears possible and could lead to new reservoirs of *C. hominis* in animals within proximity of humans, as well as cross infections to other animals (Feng et al. 2018; Widmer et al. 2020). Additionally, in Australia it was reported that some of the infected animals with *C. hominis* were also found to dwell in proximity of water sources used for drinking purposes urging to a future human health risk assessment of these resources (Zahedi et al. 2018). Finally, *C. hominis* is now deemed as also equine and non-human primate-adapted, with multiple studies revealing *C. hominis* infections in these animals (Feng and Xiao 2017; Feng et al. 2018; Hatam-Nahavandi et al. 2019; Widmer et al. 2020). Nonetheless, the genomic data of *C. hominis* obtained from equine and non-human primates appears to be different from the genome of *C. hominis* obtained from humans (Feng and Xiao 2017).

2.1.4 Zoonosis in other *Cryptosporidium* Species

Several other species of *Cryptosporidium* have also recently emerged as of relevant zoonotic concern to humans and of public health significance, namely *C. meleagridis*, *C. cuniculus*, *C. felis*, *C. canis*, *C. muris*, *C. ubiquitum* and *C. andersoni* (Ryan et al. 2016b).

Cryptosporidium meleagridis is a parasite species with low host specificity, being mainly found in birds (particularly poultry), and it is responsible for the third most cases of cryptosporidiosis observed in humans (Xiao 2010; Nakamura and Meireles 2015; Ifeonu et al. 2016; Ryan et al. 2016b). In fact, in some parts of the world this species can reach similar frequencies in human infections as *C. parvum* (Gatei et al. 2002b; Cama et al. 2007). With the development of *gp60* subtyping tools specific to *C. meleagridis* it was possible to identify 10 subtype families (IIIa–IIIj), with almost all of those also occurring in humans (Xiao and Feng 2017; Kopacz et al. 2019). Cryptosporidiosis transmission in humans might take place through anthroponotic and zoonotic routes (Cama et al. 2003; Xiao 2010; Elwin et al. 2012a; Feng and Xiao

2017), with cross-species infection of *C. meleagridis* in humans first described in a Swedish farm, where 18S DNA and 70 kDa Heat Shock Protein (Hsp70) gene sequencing of chicken and human stool samples revealed identical *C. meleagridis* sequences, indicating of zoonotic transmission (Silverlås et al. 2012). Later studies employing *gp60* subtyping confirmed the likelihood of zoonotic transmission of *C. meleagridis* between humans and birds (Stensvold et al. 2014; Wang et al. 2014; Liao et al. 2018). Besides humans and birds, *C. meleagridis* is found in a wide range of mammals, which includes hosts as diverse as rodents, great apes, marsupials, minks and cattle (Feng et al. 2007; Sak et al. 2014; Vermeulen et al. 2015; Zhang et al. 2016; Gong et al. 2017; Tan et al. 2019).

Cryptosporidium cuniculus is a recently assigned *Cryptosporidium* species, gaining taxonomic species status in 2010 after being first detected in rabbits in 1979 and genetically characterized in rabbit stool samples from the Czech Republic (Inman and Takeuchi 1979; Ryan et al. 2003a, b; Robinson et al. 2010). This species was initially depicted as host-specific, with reports of its presence in rabbits from different locations around the world (Ryan et al. 2003a; Zhang et al. 2012b; Nolan et al. 2013; Koehler et al. 2014; Liu et al. 2014b; Zahedi et al. 2016a, 2018). However, subsequent studies disproved these assumptions by linking *C. cuniculus* transmission to humans and bringing to light the zoonotic threat of this species, with the most notable case of zoonotic transmission by *C. cuniculus* taking place in the UK when a human cryptosporidiosis outbreak was attributed to a wild European rabbit (Robinson and Chalmers 2010; Cacciò and Chalmers 2016; Chalmers et al. 2019). Sporadic cases of *C. cuniculus* in humans have also been reported (Robinson et al. 2008; Molloy et al. 2010; Elwin et al. 2012a; Odeniran and Ademola 2019). With the aid of multiple loci analysis (including the 18S DNA), it was possible to differentiate this species from the genetically related *C. hominis*, and with *gp60* sequencing two subtype families were identified and described (Va and Vb) (Robinson and Chalmers 2010; Koehler et al. 2014). According to the literature, most of human infections appear to be related with the Va subtype family whilst Vb is mostly associated with infections in rabbits (Zhang et al. 2012b). More recently, the presence of *C. cuniculus* outside of humans and rabbits was documented for the first time in Australia, where an Eastern grey kangaroo and a person were both described as carrying *C. cuniculus*, though the subtype Vb (Koehler et al. 2014). This event, combined with the broad incidence of *C. cuniculus* in rabbits, might indicate a possible spill-over of *Cryptosporidium* to other animals and, therefore, also to humans (Koehler et al. 2014; Zahedi et al. 2016b). Published data from 2018 mentions *C. cuniculus* presence in alpacas located in Australia, but it was described as more of a pseudo-parasitism manifestation than a true infection (Koehler et al. 2018a). Surveillance of water catchments is also recommended in Australia as rabbits are widespread in all territory and people in rural areas are known to drink unfiltered water from water catchments (Koehler et al. 2014, 2016a; Zahedi et al. 2018).

Cryptosporidium canis and *C. felis* are species that mainly affect dogs and cats, respectively, and both species have been detected in humans from different areas of the world (Cieloszyk et al. 2012; Elwin et al. 2012b; Feng et al. 2012; Beser et al.

2015; Ebner et al. 2015; Cunha et al. 2019; Odeniran and Ademola 2019; Rojas-Lopez et al. 2020). In developing countries, *C. canis* accounts to up to 4.4% of total human cryptosporidiosis cases, whilst *C. felis* accounts to up to 3.3% of the total human cryptosporidiosis cases (Ryan et al. 2014). The host range of *C. canis* and *C. felis* is recognized as limited, with *C. canis* being detected in canids, humans, minks and a mongoose, and *C. felis* being detected in felids, humans, cattle, a rhesus monkey and a red fox (Bornay-Llinares et al. 1999; Bowman and Lucio-Forster 2010; Lucio-Forster et al. 2010; Ye et al. 2012; Jian et al. 2014; Li et al. 2015a; Zhang et al. 2016; Mateo et al. 2017; Hatam-Nahavandi et al. 2019; Odeniran and Ademola 2019). Since dogs and cats live in close proximity to humans there was always a concern about their zoonotic potential, however the risk of transmission of *Cryptosporidium* to humans seems low (Lucio-Forster et al. 2010). Studies conducted on *C. canis* and *C. felis* also showed that besides the possible zoonotic transmission of these species in humans, the anthroponotic route of infection could not be ruled out (Cama et al. 2006; Xiao 2010; Feng and Xiao 2017). Nevertheless, a probable zoonotic transmission of *C. felis* from a cat to its human caretaker was reported in 2015 after using a multiple loci analysis approach, which included 18S DNA gene, *Cryptosporidium* oocyst wall protein (COWP) gene and the Hsp70 protein gene (Beser et al. 2015). A shortcoming from this study and others in *C. canis* are related with the low discriminatory power of the above markers and lack of higher resolution tools, such as the *gp60* marker, which is considered as the golden standard when studying *Cryptosporidium* zoonotic transmission (Xiao 2010; Xiao and Feng 2017; Rojas-Lopez et al. 2020). Very recently, the first *gp60* subtyping tools were developed to *C. felis* and two suspected cases of zoonotic transmission between cats and humans were successfully verified (Rojas-Lopez et al. 2020). The only report of direct *Cryptosporidium* transmission between a dog and human could not conclusively prove if it was a zoonotic infection or a reverse zoonotic infection (Xiao et al. 2007).

Cryptosporidium muris was the first *Cryptosporidium* species ever discovered back in 1907 by Ernest Edward Tyzzer and in 1910 gained its full taxonomic species name (Tyzzer 1907, 1910). Ever since, a plethora of studies employing molecular methods found this parasite species in an array of wild animals, indicating its broad host range, which comprises mostly mammals (rodents, canids, felids, ungulates, nonhuman primates, marsupials, bats and seals) and occasionally birds (Warren et al. 2003; Santín et al. 2005; Kváč et al. 2008; Lv et al. 2009; Kodádková et al. 2010; Lucio-Forster et al. 2010; Qi et al. 2014; Sak et al. 2014; Zahedi et al. 2018; Hatam-Nahavandi et al. 2019; Tan et al. 2019; Zhao et al. 2019a). Various reports highlight its zoonotic potential after its presence being observed numerous times in humans as well as in raw and treated sewage (Guyot et al. 2001; Gatei et al. 2002a; Palmer et al. 2003; Ghenghesh et al. 2012; Hasajová et al. 2014; Spanakos et al. 2015; Martins et al. 2019). Further research performed on healthy adults to assess *C. muris* human infectivity also reinforced the zoonotic potential of this species (Chappell et al. 2015). In this study, six healthy adults were infected with *C. muris* and examined for six weeks for infection/disease (Chappell et al. 2015). All volunteers became infected with *Cryptosporidium* and two of the volunteers displayed self-limiting diarrhoea disease (Chappell et al. 2015).

Cryptosporidium andersoni is a parasitic species mainly encountered in cattle and it is described as more frequent in post-weaned, juveniles and adult cattle (Gong et al. 2017; Thomson et al. 2017). However, its presence in other wild and domestic animals has been increasing in recent years with numerous studies reporting its incidence in other ungulates, rodents, pandas (both giant and red), Asiatic black bears and rhesus monkeys (Lv et al. 2009; Du et al. 2015; Wang et al. 2015a, 2020, b; Hatam-Nahavandi et al. 2019; Wu et al. 2019). *Cryptosporidium andersoni* frequency in humans has been documented in a small number of countries, with most of the cryptosporidiosis cases so far being sporadic (Leoni et al. 2006; Waldron et al. 2011; Agholi et al. 2013; Hussain et al. 2017). However, in China its prevalence is unusually high with two studies (Jiang et al. 2014; Liu et al. 2014a) attributing to *C. andersoni* most of the *Cryptosporidium* infections in humans and another study stating *C. andersoni* as responsible for the second most occurrences of cryptosporidiosis manifestation in humans (Liu et al. 2020). Furthermore, in China it was discovered that *C. andersoni* is routinely found in drinking source water adding more evidence to its importance as a cause of cryptosporidiosis in humans and that cattle might be the principal origin of the parasite (Feng et al. 2011; Xiao et al. 2013; Hu et al. 2014). The full extent of the zoonotic potential of this species is still unknown and thus more molecular studies are required to fully comprehend its transmission dynamics in humans (Liang et al. 2019; Liu et al. 2020).

Cryptosporidium ubiquitum, is a species classified as an emerging zoonotic pathogen after being associated with multiple cases of cryptosporidiosis in humans in different parts of the world (Li et al. 2014). Its status as of public health concern stems from *C. ubiquitum* ample geographic distribution and wide host range (Li et al. 2014). Besides humans, *C. ubiquitum* presence has been observed in domestic and wild ungulates, primates, rodents, marsupials, hedgehogs, carnivores and birds (Koehler et al. 2016b; Li et al. 2016; Kellnerová et al. 2017; Mateo et al. 2017; Zahedi et al. 2018; Zhao et al. 2018; Chen et al. 2019a; Hatam-Nahavandi et al. 2019; Kubota et al. 2019). After the development of *gp60* subtyping tools specific for *C. ubiquitum*, eight subtype families have been described (XIIa–XIIh) and host adaptations, as well as infection/transmission dynamics, have been uncovered (Li et al. 2014; Kubota et al. 2019). The subtype XIIa is linked to ruminants found worldwide, the subtypes XIIb–XIId linked to rodents based in the USA, and subtypes XIIe and XIIf linked to field mice from Slovakia (Li et al. 2014). However, recent phylogenetic analysis based on 18S DNA, actin and COWP protein gene sequences show that these genotypes are distinct from *C. ubiquitum* and belong to *Cryptosporidium* apodemus genotype I and II. Therefore, in accordance with the *gp60* subtyping nomenclature (Lv et al. 2009; Sulaiman et al. 2005), subtype XIIe is now XVIIa for *Cryptosporidium* apodemus genotype I, and XIIf is XVIIIa for *Cryptosporidium* apodemus genotype II (Čondlová et al. 2019). The rodent subtypes XIIb–XIId are the source of human infections in the USA, whilst in the other areas of the world the ruminant subtype XIIa is the main culprit for human infections (Li et al. 2014). An additional application of *gp60* subtyping tools specific for *C. ubiquitum* allowed to reveal its incidence in water bodies and assess its role in human cryptosporidiosis (Li et al. 2014; Huang et al. 2017). A waterborne route of transmission of

C. ubiquitum to humans in the USA was suggested after the *C. ubiquitum* rodent subtypes XIIb and XIId were also found in drinking source water (Li et al. 2014). Since these subtypes are also commonly found in humans living in the USA, drinking untreated water contaminated by infected wildlife might be responsible for the *Cryptosporidium* transmission (Li et al. 2014). Finally, in 2018 *C. ubiquitum* was found in urban wastewater from China and, after subtyping with *gp60*, the XIIg and XIIh subtypes were identified as the ones present in the wastewater (Huang et al. 2017). Additional phylogenetic analysis clustered these subtypes with the USA rodent subtype families, and researchers hypothesized that human infections in China due to *C. ubiquitum* could be caused by parasites of rodent origin, as it was observed in the USA (Huang et al. 2017).

Another species of *Cryptosporidium*, termed *C. viatorum*, has been touted as a potential emergent zoonotic pathogen (Koehler et al. 2018b). *Cryptosporidium viatorum* is a parasite first discovered in 2012 and initially described as host-specific in humans, with numerous reports in humans across the globe (Elwin et al. 2012b; Stensvold et al. 2015a; Chen et al. 2019b; Xu et al. 2020). After subtyping analysis with *gp60* gene sequence, four subtype families were found (XVa to XVd) and 13 subtypes have been defined so far with the following terminology XVaA3a–h, XVaA6, XVbA2G1, XVcA2G1a, XVcA2G1b and XVdA3 (Xu et al. 2020). The subtypes belonging to the XVa family were the first subtypes to be reported and initially described as only occurring in humans or in wastewater (Stensvold et al. 2015a; Huang et al. 2017). However recent studies observed new subtypes of *C. viatorum* (XVbA2G1, XVcA2G1a, XVcA2G1b and XVdA3), as well as subtypes formerly associated only with humans (XVaA6, XVaA3g and XVaA3h) in broadly distributed wild rats from Australia and China (Koehler et al. 2018b; Chen et al. 2019b; Zhao et al. 2019a). This new data suggested that a high prevalence and distribution of *C. viatorum* among wild rats, coupled with living in close proximity to humans, could play a role in the dissemination of *Cryptosporidium* and cause a risk to human health (Koehler et al. 2018b; Xu et al. 2020).

As a result of the increased human intrusion into the environment and its subsequent effect on wildlife, more and more *Cryptosporidium* species/genotypes are expected to emerge as human pathogenic agents in the future (Ryan et al. 2016b). Examples of those include the chipmunk genotype I, *C. erinacei* and more recently *C. occultus* (Ryan et al. 2016b; Zahedi et al. 2016b; Zhao et al. 2019a; Xu et al. 2020).

3 Developmental Life Cycle of *Cryptosporidium*

Cryptosporidium has a complex monoxenous life cycle, consisting of both asexual and sexual stages (Fig. 2). The cycle begins upon the ingestion of oocysts by a suitable host. These oocysts are a thick-walled, double-layered structure encasing four sporozoites (Ryan and Hijjawi 2015). This structure is highly resistant to both chemical and mechanical disruptions (Fayer and Ungar 1986), thus providing a

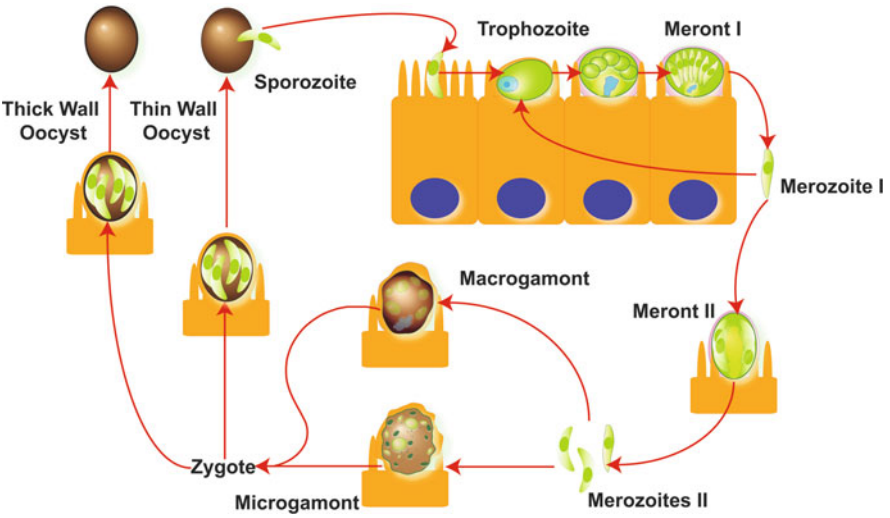

Fig. 2 The life cycle of *Cryptosporidium* species. Upon consumption of the oocyst, typically through drinking water, the parasite enters the gut of the soon-to-be host and undergoes the process of excystation. At this point 4 sporozoites, each haploid, emerge from the approximately 5–6 μm diameter oocyst and proceed via a gliding motility to interact with and invade host epithelial cells. Current understanding of this stage of infection holds that the invasion process is a combination of apical complex mediated penetration of host membrane and host cell mediated endocytosis. Reproduced from Miller et al. (2018)

robust form of protection to the more fragile sporozoites within and subsequently maintaining their viability. Once ingested, the oocysts encounter a range of environmental cues within the host, such as temperature, pH, bile salts, pancreatic enzymes and CO_2 (Fayer and Leek 1984; Reduker and Speer 1985; Robertson et al. 1993; Hijjawi et al. 2001), which could trigger excystation. This process leads to the release of four motile sporozoites in the infection sites (small and large intestines, stomach, bursa of Fabrici or lungs) of the host, through a "suture" in the oocyst wall (Reduker et al. 1985a, b), which carry on to invade the epithelial lining (Thompson et al. 2005; Borowski et al. 2008; Ryan and Hijjawi 2015). Attachment and invasion of sporozoites to host epithelial cells is facilitated by the release of the contents of the apical complex of sporozoites (rhoptries, micronemes, and dense granules) (Ward and Cevallos 1998; Blackman and Bannister 2001), and during this process protrusions form in the apical membrane of the host cell that encapsulate the parasite forming the parasitophorous vacuole (Ward and Cevallos 1998; Thompson et al. 2005). Whilst this structure is common among many other Apicomplexan parasites, including *Toxoplasma gondii* and *Plasmodium falciparum* (O'Hara and Chen 2011), in *Cryptosporidium* the parasitophorous vacuole creates a unique position in between the cytoplasmic membrane and the apical membrane of the host cell, resulting in an intracellular but extracytoplasmic location (Bones et al. 2019). Formation of the parasitophorous vacuole is accompanied by formation of the 'feeder organelle', a tube that connects the parasite to the cytoplasm of the host cell

and has been hypothesized to be involved in the transport of nutrients and energy uptake from the host to the parasite (Huang et al. 2004a; Bones et al. 2019).

After invasion, the asexual life cycle continues with the differentiation of sporozoites into trophozoites, which undergo nuclei division by merogony, forming a "type I" meront, each containing six to eight "type I" merozoites. Once it reaches maturation, "type I" merozoites are released and go on to infect other host cells where they will differentiate again into either a "type I" meront or a "type II" meront, the latter of which produces four "type II" merozoites. The sexual stage of *Cryptosporidium*'s life cycle progresses from "type II" merozoites, which develop into macrogamonts or microgamonts after invading new host cells. Microgamonts form 14–16 microgametes, non-flagellated forms that are released from the parasitophorous vacuole and fertilize the macrogamont to form a zygote. Once formed, the zygote develops into one of two types of oocyst: thin-walled oocysts, which are responsible for auto-infective cycles by excysting within the intestinal lumen of the same host (O'Donoghue 1995; Thompson et al. 2005); or thick-walled oocysts, which is the environmentally resistant form excreted in the faeces of the host into the environment, re-starting the cycle by infecting another susceptible host (s) (Arrowood 2002; Ryan and Hijjawi 2015). The ability of *Cryptosporidium* to re-infect the same host is a characteristic not observed in other coccidian parasites (Tzipori and Ward 2002). This ability is attributed to the recycling of "type I" meronts, as well as for the development of thin-walled oocysts, both of which have been implicated as the reason for chronic cryptosporidiosis in immunocompromised hosts (O'Donoghue 1995).

Despite the difficulties hindering research of *Cryptosporidium* biology (see Sect. 6), research efforts by Borowski and colleagues (Borowski et al. 2010) provided the first characterization of *C. parvum* life-cycle stages in an in vitro system with human ileocecal epithelial cell line (HCT-8) using scanning electron microscopy (SEM), and providing a detailed time-line and morphological characterization of the developmental stages. In this work, the authors observed that the life cycle was completed within 96 h of infection with *C. parvum* oocysts, with trophozoites observed at six hours post-infection, type I meronts and type I merozoites appearing at 24 h, type II meronts and type II merozoites visualized 72 h post-infection, and gametes being found at 96 h post-infection. This study was consistent with previous observations made for *C. parvum* (Hijjawi et al. 2001) and *C. andersoni* (Hijjawi et al. 2002) HCT-8 infection using light microscopy, as well as with data obtain in vivo (Valigurová et al. 2008). Over the last few years, new studies have presented new in vitro 2D systems for culturing and observing the biology of the parasite in the lab (Miller et al. 2018; for review see Bones et al. 2019), which allowed further dissection of the biology and life-cycle of *Cryptosporidium* species.

4 Morphological Description of *Cryptosporidium* Species

Despite a significant shift to molecular and immunological methods for the detection of pathogens, light microscopy is still an essential diagnostic tool in parasitology; electron microscopy is also necessary, but mostly as a research tool rather than for diagnostics. Currently, there are 45 recognized *Cryptosporidium* species and over 100 genotypes, which most likely represent different species (Holubová et al. 2020). The majority of the recognized species have been supported by morphology and morphometric data of oocysts (Table 2), a few by data of endogenous stages (Table 3), but unfortunately, for most of the genotypes, these data are still lacking (Feng 2010; Kváč et al. 2014b; Nakamura and Meireles 2015; Čondlová et al. 2019).

During their life cycle, parasites of genus *Cryptosporidium* form a number of morphologically and morphometrically developmental stages (see Sect. 3; Fig. 1). The infected host releases exclusively oocysts to the environment, which go on to infect other susceptible hosts. Other developmental stages such as trophozoites, merozoites and gamonts never leave the host and their detection is possible only in the tissue where an ongoing infection occurs. There is large number of different diagnostic methods for the direct detection of *Cryptosporidium* spp.

4.1 Oocysts

Oocysts can be detected directly in faeces/stool by bright-filed (BF), phase-contrast (PC), or by using differential interference contrast (DIC) microscopy. Oocysts can be observed directly after diluting the faeces/stool with a suitable liquid (e.g. water) that does not change the morphology of the oocysts and does not affect the quantification methods that are subsequently and often used (Smith 2008). The oocyst wall is thick-walled, smooth and colorless, lacks morphological structures such as sporocyst, micropyle and polar granules (Thompson et al. 2005). The inner structure is hardly observed by BF microscopy. To observe sporozoites and residual bodies the DIC or PC microscopy is more suitable (Fig. 2). Morphometrical and morphological description of oocysts should be taken at the 1000X magnification and should only be observed in a suspended liquid that does not cause the distortion due to excystation, expansion, contraction or disintegration.

Various staining methods are often used to detect *Cryptosporidium* oocysts in faecal samples, including Giemsa, acid-fast Ziehl-Neelsen, Auramine-O, aniline-carbol-methyl violet staining and negative staining with strong carbol-fuchsin (Tyzzer 1910; Henriksen and Pohlenz 1981; Miláček and Vítovec 1985; Ley et al. 1988; Casemore 1991). During the fixation procedure of the wet smear, the oocyst partially loses its original shape and, as a result, a subsequent measurement may provide inaccurate data. On the other hand, the staining can highlight the internal structures, which are poorly observable in BF microscopy (Figs. 3 and 4). After staining, oocysts appear as spherical (intestinal species) or oval/ovate (gastric

Table 3 Comparison of the developmental stages of *Cryptosporidium baileyi*, *C. meleagridis*, *C. ornithophilus*, *C.proliferans*, *C. reichenbachklikei* and *C. cichlidis* (Slavin 1955; Current et al. 1986; Paperna and Vilenkin 1996; Melicherová et al. 2014; Holubová et al. 2020)

	C. baileyi	*C. meleagridis*	*C. ornithopohilus*	*C. proliferans*	*C. reichenbachklikei*	*C. cichlidis*
Developmental stage	Average size (μm)					
Sporozoite	5.8 × 1.1	×	6.07 × 0.59	11.72 × 0.98	3.6–4.0 × 0.4–0.5	3.6–4.1× 0.3–0.9
Trophozoite	Unknown	4.0–5.0	4.36 × 3.90	7.85 × 4.89	Unknown	Unknown
Meront	5.0–5.2 × 4.9–5.1	5.0 × 4.0	5.96–7.50 × 5.12–6.48	9.51 × 7.25	2.7–3.4 × 2.2–3.0	2.1–3.7 × 2.4–3.1
Merozoite	5.0–6.9 × 1.1	5.0 × 1.1	5.05 × 0.77	9.64 × 1.52	1.6–2.2 × 0.45–1.4	3.5 × 0.6–0.9
Microgamont	4.0 × 4.0	4.0 × 4.0	6.54 × 6.39	6.18 × 5.54	2.4 × 1.8	3.1–4.4 × 2.8–3.8
Macrogamont	5.8 × 5.8	5.0 × 4.0	6.70 × 6.10	8.04 × 6.49	2.8–3.3 × 1.7–2.7	3.9–4.6 × 2.2–3.4
Unsporulated oocyst	6.3 × 5.2	4.5 × 4.0	6.14 × 5.20	Unknown	Unknown	Unknown
Oocyst	6.2 × 4.6	4.5–6.0 × 5.0–6.0	6.13 × 5.21	7.7 × 5.3	2.4–3.18 × 2.4–3.0	4.0–4.7 × 2.5–3.5

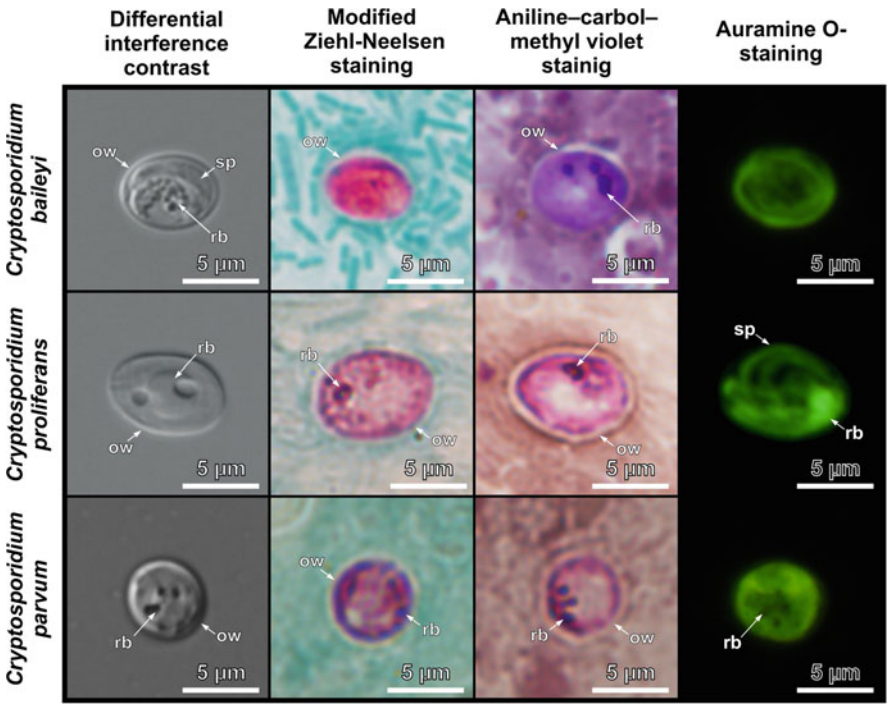

Fig. 3 Oocyst of *Cryptosporidium baileyi*, *Cryptosporidium proliferans* and *Cryptosporidium parvum* visualized in differential interference contrast microscopy and stained by modified Ziehl-Neelsen (Henriksen and Pohlenz 1981), aniline–carbol–methyl violet (Miláček and Vítovec 1985) and auramine O (Ley et al. 1988). *ow*—oocyst wall; *sp*—sporozoite; *rb*—residual body. Scale bar included in each picture

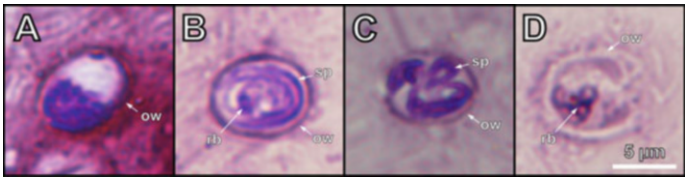

Fig. 4 Oocysts of *Cryptosporidium proliferans* visualized in aniline-carbol-methyl violet staining (Miláček and Vítovec 1985). (**a**) partially stained oocysts—most often visible during staining. (**b**) oocyst with visible sporozites—rarely observed. (**c**) oocysts with almost excysted sporozoites—very rarely. (**d**) empty oocyst with residual body—sporadically observed. Scale bar included

species) objects bordered by a thin unstained ring (oocyst wall). The internal content of the oocyst is stained in whole or in part (Fig. 4a). Residual bodies are stained very well and are seen often, whilst free sporozoites are rarely observed (Fig. 4b, c). The highlighted sporozoites can be observed in older samples where the oocysts disintegration occurs, or in samples that have been exposed to excystation factors (Widmer et al. 2007). In this case, the oocyst shell can also be observed in stained smear (Fig. 4d).

Most of the oocysts are similar in shape and overlap in size as well (Table 2). Their size, shape and staining characteristic can be helpful to distinguish from other microscopic objects but generally it is hard, and even in many cases impossible, to determine the actual *Cryptosporidium* species (Table 2). The oocysts of *Cryptosporidium* spp. that inhabit the intestine have a spherical shape and usually measure from 4 × 6 μm, however (e.g. oocyst of *C. baileyi*), the bird-specific species, measure 6.3 × 5.2 μm and has elliptical shape (Current et al. 1986). Most *Cryptosporidium* spp. that infect the stomach of their hosts have larger oocysts measured 7.5 × 6.5 μm, with ovoid or ellipsoid shape; the exception to this is *C. testudinis*, the species specific for tortoises, which is also phylogenetically clustered to gastric species, but has oocysts more similar to the intestinal than the gastric species (Tyzzer 1910; Lindsay et al. 2000; Ježková et al. 2016; Kváč et al. 2016).

Although mixed infections are relatively common, it is usually impossible to differentiate such an infection based on the oocyst morphometry. Only when the difference in the size and shape of the oocysts is significant is it probable to estimate what the actual species/genotypes are. For example, the mouse is the typical host of *C. tyzzeri* and *C. muris*, which can be distinguished very well from each other (Kváč et al. 2012; Ren et al. 2012). However, the mouse is a minor host of other intestine species, such as *C. hominis* or *C. parvum*, which can also be mistaken for *C. tyzzeri* (Kváč et al. 2012). Similarly, a calf could be infected with *C. andersoni*, *C. parvum*, *C. ryanae* and *C. bovis* at the same time, but only *C. andersoni* can be reliably distinguished from others (Santín and Zarlenga 2009). Another example occurs in pigs, where *C. suis* is theoretically distinguishable from *C. scrofarum*, but in common routine diagnosis differentiation is very difficult (Vítovec et al. 2006; Kváč et al. 2013).

Faeces/stool specimens can be stored in 10% formalin, sodium acetate-acetic acid formalin, polyvinyl alcohol (PVA), potassium dichromate, RNA later and water or left without fixation. Specimens fixed with PVA are not suitable for staining methods. If specimens are stored in a preservative solution or in water to moisture a dry sample, the final differentiation of oocysts is less intensive when aniline-carbol-methyl violet staining is used (Kváč and Hůzová 2018).

The success of the detection also depends on the number of oocysts in the examined sample. Samples from symptomatic cases often contain a large number of oocyst, which could be easily detected using a direct smear. However, most of naturally infected domestic and wild animals often lack clinical signs and the intensity of infection is very low with intermittent excretion of oocysts (Chelladurai et al. 2016; Ježková et al. 2016; Čondlová et al. 2018, 2019; Kváč et al. 2018). When the infection intensity is less than ~2000 oocyst per gram of stool/faeces the staining method is not effective, which could give false negative results and, thus, it is better to use a concentration method (Kváč and Hůzová 2018).

4.2 Endogenous Life Stages

The size of the endogenous developmental stages has been reported only in a few *Cryptosporidium* spp. (Table 3). Similarly to the oocyst's size, the size of developmental stages overlays among species and it cannot be use for species determination. Despite the fact that thin-walled oocysts do excyst in the host (see Sect. 3), their detection in faeces/stools or sputum would be very difficult due to their small size and fragility. Endogenous life stages are not found in the stool/faeces of the infected host. For their detection, using necropsy or biopsy followed by histological methods, it is necessary to investigate tissue samples from an infected organ by staining of mucosal smears or by electron microscopy.

In histological sections it is either not possible or very difficult to distinguish the individual types of developmental stages (Fig. 5). Histological sections are often stained with hematoxylin and eosin, Periodic Acid-Schiff stain or Wolbach's modified Giemsa stainings (Jirků et al. 2008; Robinson et al. 2010; Ren et al. 2012; Kváč et al. 2014a; Holubová et al. 2019, 2020). The developmental stages appear as a light to dark purple object connected to the epithelial cells in the microvillus border (Fig. 5). Similarly, scanning electron microscopy (SEM) observations is not the most suitable technique to differentiate various developmental stages. The stages are mostly hidden under the parasitophorous vacuole and only rarely can you observe the vacuole rupture and the subsequent uncovering of the internal morphological features (Fig. 6).

The staining of mucosal smears (e.g. Wright's staining; Fig. 7a–f) and transmission electron microscopy (TEM; Fig. 7g–i) can be used to differentiate developmental stages in smears and tissue sections, respectively (Tyzzer 1912; Holubová et al. 2020). In the staining of mucosal smears and TEM sections, the developmental stages are mostly enveloped by a parasitophorous vacuole, which appears as an unstained halo in Wright's staining (Fig. 7). As previously mentioned, free sporozoites are rarely detected. Trophozoites appear as round to spherical uninuclear forms and showed high variability in size (Melicherová et al. 2014; Holubová et al. 2020). The early trophozoites could be two to three times smaller than late ones. Type I meronts contain usually eight merozoites [six found in *C. proliferans* (Melicherová et al. 2014)] and Type II meronts containing four merozoites. Type III meront with eight merozoites was observed only in *C. baileyi* (Current et al. 1986). Free merozoites are also infrequently found (Fig. 7). Microgamonts containing 16 microgametes are observed more rarely than macrogamonts, typified by a number of amylopectin granules in their cytoplasm and a foam-like appearance. Zygotes are lightly stained compared to the unstained oocysts (Holubová et al. 2020).

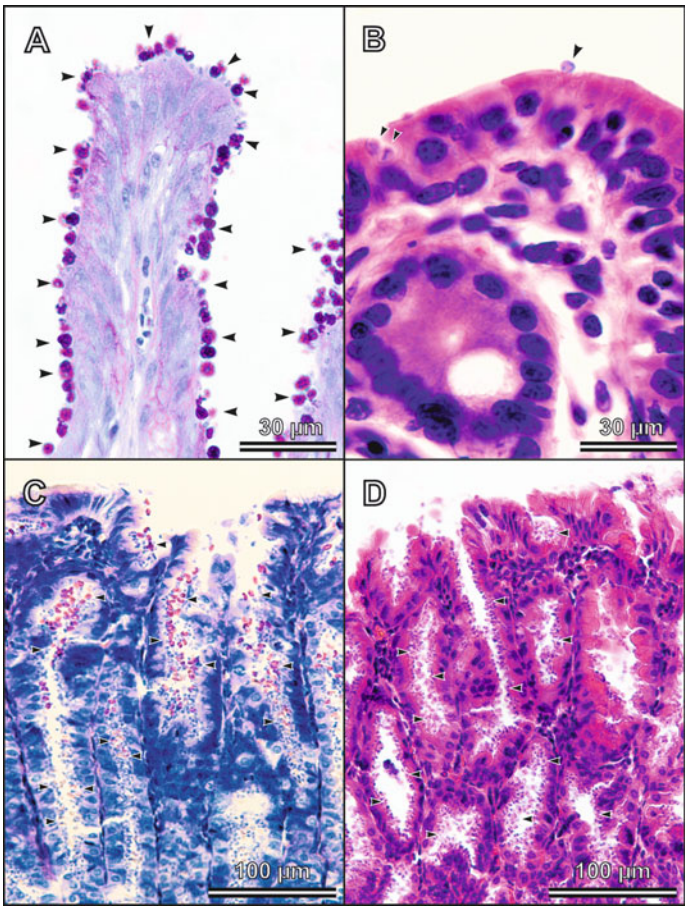

Fig. 5 Histological section (**a**) Developmental stages of *Cryptosporidium baileyi* in epithelium of the trachea from an experimentally infected chicken (*Gallus gallus* f. *domestica*) with *Cryptosporidium baileyi*, stained with Periodic Acid–Schiff stain. (**b**) Developmental stages of *Cryptosporidium microti* in epithelium from the colon of an experimentally infected common vole (*Microtus arvalis*), stained with Haematoxylin-Eosin stain. (**c**) Developmental stages of *Cryptosporidium proliferans* in glands of glandular part of stomach of an experimentally infected Southern multimammate mouse (*Mastomys coucha*), stained with Wolbach's modified Giemsa stain. (**d**) Developmental stages of *Cryptosporidium proliferans* in glands of glandular part of stomach of an experimentally infected Southern multimammate mouse (*Mastomys coucha*), stained with Haematoxylin-Eosin stain. Bar included in each picture

5 *Cryptosporidium* 'Omics

Analyzing the gene expression and the generation of metabolic profiles of *Cryptosporidium* during its life-cycle is a crucial step to understand the pathogenicity of this parasite. However, such studies have been hampered by the lack of a robust in vitro

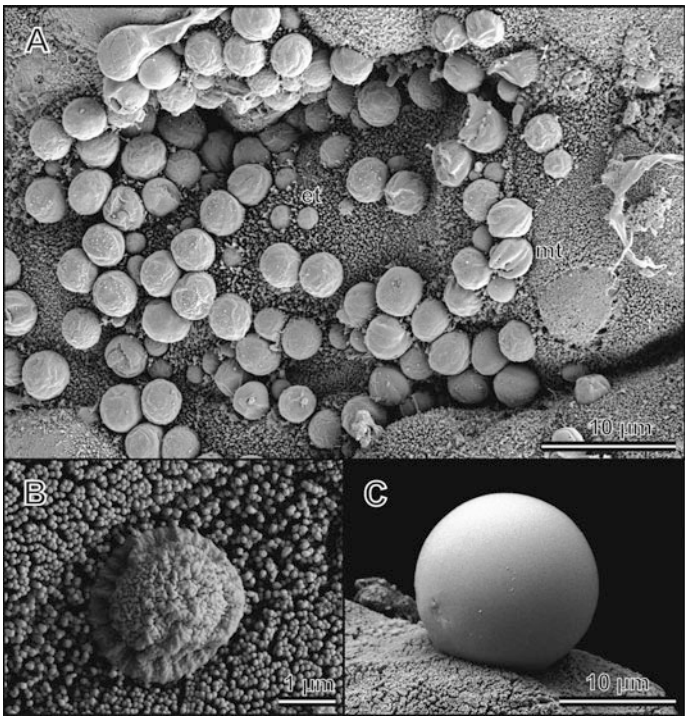

Fig. 6 Scanning electron microphotograph of developmental stages of *Cryptosporidium ornithophilus*. (**a**) Cross-section of a colon covered with various developmental stages of *C. ornithophilus* in experimentally infected chicken. Early trophozoites (et) and a meront with four visible merozoites (mt). (**b**) Detail of early trophozoite (2.8 × 2.4 µm). (**c**) Detail of a developmental stage (15.8 × 15.0 µm). Bar included in each picture

culture system capable of sustaining *Cryptosporidium*'s complete development in addition to (until recently) the lack of genetic manipulation tools. Furthermore, isolation of *Cryptosporidium*'s intracellular development forms from the host cell has not yet been achieved, making it difficult to assess and validate the expression of stage-specific genes and proteins. Despite these limitations, some transcriptomic and proteomics studies have been carried out in *C. parvum*, which will be summarized and discussed herein.

5.1 Transcriptomics

Transcriptomic studies allow us to identify expression-level changes in genes of an organism at different time-points and/or experimental conditions by analyzing the sum of its RNA transcripts. One of the first transcriptomic studies carried out in

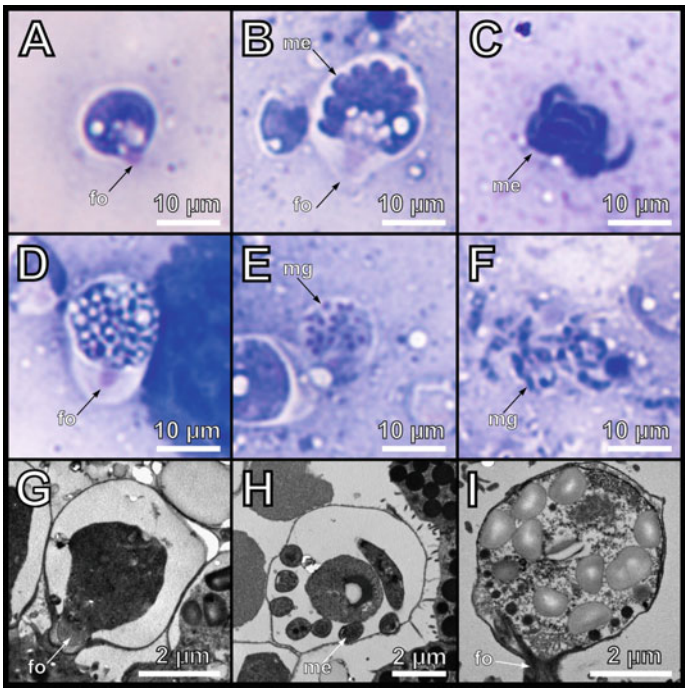

Fig. 7 Developmental stages of *Cryptosporidium proliferans* obtained from the stomach of mouse (*Mus musculus*) experimentally infected with 1,000,000 oocysts and sacrificed 20 days post infection and stained with Wolbach's modified Giemsa staining (**a**–**f**) or observed under transmission electron microscope (**g**–**i**). (**a**) mononuclear trophozoite with well visible feeder organelle (fo), (**b**) Type I meront with eight forming merozoites and feeder organelle (fo), (**c**) eight free merozoites, (**d**) macrogamont visible feeder organelle (fo), (**e**) microgamont with small compact nuclei localized at the cytoplasm, (**f**) a group of free microgametes, (**g**) mononuclear trophozoite inside of parasitophorous vacuole with visible feeder organelle (fo), (**h**) mature Type I meront with eight merozoites, (**i**) macrogamont connected to epithelium by feeder organelle (fo). Scale bar included in each picture

C. parvum used RT-qPCR to assess the transcription level for 3302 genes at seven different time points, during the course of in vitro infection of HCT-8 cells (Mauzy et al. 2012). Mauzy et al. (2012) identified a total of nine differentially-expressed clusters and 18 unequally distributed functional categories spanning the 72 h infection course. Excysted sporozoites (2 h after infection) exhibited the lowest number of genes being expressed compared to all other time points, however expression levels of transporter genes were increased, consistent with the necessity for this parasite to access the host cell contents to offset the absence of pathways involved in energy production (beyond glycolysis) and *de novo* synthesis of amino acids. The heavy dependence on host cell-acquired nutrients was further illustrated at 6 h post-infection (trophozoite stage) with an increase in expression of genes encoding components of the proteasome complex which can degrade parasite- or host-derived proteins for recycling of amino acids. An increase in expression of other genes

involved in protein translation, including chaperones, and DNA replication was also observed at this time point, and correlated with the preparation of the trophozoite for mitoses and development into meront I. Interestingly, the expression profile observed at the 2 h time point (sporozoites) and at the 24 h time point (meront I/merozoites) revealed very different transcriptomic profiles, with DNA-associated genes involved in replication and mitosis being over-represented at 24 h. The different transcriptomic profiles of sporozoites and merozoites suggests that even though both are the forms responsible for active invasion of epithelial cells, they appear to be biochemically distinct from one another. The onset of the sexual stage of development at 48 h through 72 h post-infection is accompanied by an increase in the expression of metabolic enzymes, which suggests a shift in the metabolic need of the parasite (Mauzy et al. 2012). Shortly after, Zhang et al. (2012a) developed a *C. parvum*-specific microarray to study the gene expression of untreated and UV-treated oocysts. The transcriptome of untreated oocysts revealed half (51%) of the total number of genes is expressed during this developmental stage. Assignment of genes into functional categories revealed that oocysts are highly active in protein synthesis, with genes involved in ribosome biogenesis (13.8%), gene expression (12.8%) and RNA metabolism (14.3%) being among the most highly expressed. Furthermore, components of the proteasome complex were also highly expressed, suggesting that during the oocyst developmental stage this parasite relies on protein degradation to recycle amino acids and compensate for the lack of pathways which would allow this parasite to synthesize nutrients (Abrahamsen et al. 2004; Thompson et al. 2005; Rider Jr. and Zhu 2011) and offers a possible explanation on how oocysts can remain viable for long periods of time in the environment prior to infecting and scavenging nutrients from the host cell.

Both transcriptomic approaches discussed above have the advantage of being simple, relatively low-cost, and well-established approaches. However, both microarrays and RT-qPCR rely on prior knowledge of the genome sequence for design of the oligonucleotide probes, and the dependency on probes for the detection of transcripts adds further issues relating to poor probe design. RNA-seq is a relatively new technology that circumvents these issues, making it suitable to study the transcriptome of organisms whose genome has not yet been sequenced; it exhibits a higher sensitivity than either microarrays or RT-qPCR as it depends on sequence coverage rather than detection of fluorescence; and allows for the simultaneous study of host and parasite transcriptome. Lippuner et al. (2018) used comparative analysis to analyse the transcriptome of oocyst-purified sporozoites, in vitro-cultured *C. parvum*, and *C. parvum* purified from the intestines of experimentally infected calves. The analysis revealed a higher metabolic activity in in vivo intracellular stages of the parasite compared to that expressed in sporozoites alone, supporting observations previously made showing expression of transporters, DNA-associated, and transcription-related genes at the sporozoite stage, but not expression of metabolism-related genes, whose expression was increased at the meront (12 h post-infection) and sexual cycle (48 h and 72 h post-infection) stages (Mauzy et al. 2012). Both observations are suggestive of the quiescent state of the sporozoites, which are already packed with the machinery and proteins necessary for

invasion and lie dormant within the oocyst until activation (Snelling et al. 2007; Sanderson et al. 2008; Lendner and Daugschies 2014). Several mucin proteins, including gp900 and gp40/15, were expressed mainly in vivo and, to a lesser extent, in sporozoites, to where these proteins have previously been localized to (Petersen et al. 1992; Barnes et al. 1998; Cevallos et al. 2000a, b; O'Connor et al. 2007), which further supports that sporozoites are primed for invasion (Snelling et al. 2007; Sanderson et al. 2008; Lendner and Daugschies 2014). Interestingly, genes encoding oocyst wall proteins (COWPs) were expressed both in vivo and in vitro. The expression of COWPs in vitro raises further questions on the reason why *C. parvum*-infected HCT-8 cells are unable to or produce a very low yield of oocysts (Hijjawi et al. 2001; Thompson et al. 2005; Hijjawi 2010; Lippuner et al. 2018).

Despite the limitation of working with *Cryptosporidium*, the transcriptomic studies carried out by Mauzy et al. (2012), Zhang et al. (2012a), and Lippuner et al. (2018), as well as any future studies of this nature, contribute with invaluable data on the pathogenicity of this parasite.

5.2 Proteomics

Transcriptomic analysis provides a good snap-shot of the main pathways being expressed in certain conditions or life stages of *Cryptosporidium*. However, the transcriptome of an organism does not correlate with protein abundance and activity, and other factors, such as translation efficiency, protein stability, mRNA degradation, post-translational modifications, and protein interactions, all have a role in establishing the complexity of the proteome. As such, proteomic studies are necessary to acquire a global understanding of the different cellular processes and how they are integrated. In *Cryptosporidium*, global proteomic profiling studies are scarce and most have focused on the proteome of oocyst and sporozoites of *C. parvum*, as they are the parasite forms most easily obtain in high enough quantity and purity.

Snelling et al. (2007) used mass spectrometry to identify the total proteome in non-excysted and excysted sporozoites. Their approach identified a subset of 26 proteins whose expression was increased in excysted sporozoites, compared to non-excysted. This subset included ribosomal proteins and heat-shock proteins (Hsp70 and Hsp90), both of which were suggested to increase due to the necessity for this parasite to rapidly initiate protein synthesis after the dormancy period characteristic of the oocyst stage. Four metabolic enzymes were also identified among the subset of proteins with significant increased expression after excystation, all of which are involved in glycolysis. The lack of TCA cycle and cytochrome-based respiratory chain in *C. parvum* suggests that this parasite relies on glycolysis for energy production (Abrahamsen et al. 2004), hence the identification of these proteins was not wholly unexpected. What is interesting is that previous transcriptomic studies have only identified the mRNA transcript of one of these metabolic enzymes—i.e. lactate dehydrogenase—in oocysts and free sporozoites

(Zhang et al. 2012a). Whether these discrepancies relate to the inability of transcriptomic studies to fully reflect the protein content of an organism, are due to differences in oocyst and sporozoite handling procedures, or if they reflect any functional significance remains to be determined.

In 2013, Siddiki published a study on the proteome of excysted sporozoites of *C. parvum* using 1D SDS-PAGE to obtain the whole protein repertoire (soluble and insoluble proteins), as an alternative to two-dimensional electrophoresis, a more labor intensive and time-consuming approach that presents limitations when resolving membrane proteins (Siddiki 2013). This alternative approach was successful at separating the entirety of the proteins obtained from excysted sporozoites, and allowed the identification of 33 *C. parvum* unique proteins distributed among six functional categories, the most prevalent being protein biosynthesis (49%), hypothetical proteins (30%), and energy metabolism (9%). The prevalence of proteins involved in protein biosynthesis (e.g. ribosomal proteins) is consistent with observations from previous work by Snelling et al. (2007). The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase was also identified in both studies (Snelling et al. 2007; Siddiki 2013), further supporting the hypothesis that glycolysis is the main pathway for energy production in *C. parvum*.

In another proteomic study, Sanderson et al. (2008) identified 30% of the predicted proteome in excysted sporozoites, in which several proteins have been implicated in adhesion and invasion of the host cell. In apicomplexan parasites, proteins involved in attachment and invasion are localized to the organelles of the apical complex—rhoptries, micronemes, and dense granules—from which they are successively secreted following initial host-parasite interactions (Tzipori and Ward 2002; Smith et al. 2005; Wanyiri and Ward 2006; Borowski et al. 2008). Searching for orthologues in *T. gondii* and *P. falciparum* revealed that *C. parvum* possesses 24 putative micronemal proteins and 38 putative rhoptry-associated proteins, 14 and 12 of which were identified in the proteome of excysted sporozoites, respectively (Sanderson et al. 2008). Among the putative micronemal proteins identified in *C. parvum* were TRAP-C1 (Spano et al. 1998; Wanyiri and Ward 2006; Boulter-Bitzer et al. 2007), P23 (Arrowood et al. 1991; Perryman et al. 1996), gp900 (Petersen et al. 1992; Barnes et al. 1998), and gp15/40 (Cevallos et al. 2000a, b; Strong et al. 2000; O'Connor et al. 2007), glycosylated mucin-like proteins previously characterized and suggested to have a role in attachment and invasion to the host cell (Table 4).

Whilst they provide a very good insight into the biology of *Cryptosporidium*, global proteomic studies are often expensive, labor intensive, complex and, in the particular case of *Cryptosporidium*, only feasible for the oocyst and sporozoite life stage as isolation of the intracellular stages from the host cell at a high enough quantity and purity is not presently possible. However, the importance of *Cryptosporidium* as a human pathogen has had tremendous weight, and the need for an anti-cryptosporidial drug or vaccine has prompt researchers for many years to identify potential drug targets in *Cryptosporidium*, most of which have been suggested to be involved in excystation, attachment, or invasion, and are summarized in Table 4. The combination of proteomics with the recent advances in in vitro propagation methods

Table 4 Summary of putative *Cryptosporidium* spp. proteins involved in attachment and invasion

Protein	Putative role(s)	Characteristics	Reference(s)
Serine Protease	Excystation	–	Forney et al. (1996)
Aminopeptidase	Excystation	–	Okhuysen et al. (1996)
CSL	Adhesion	~1300 kDa soluble protein; Affinity for an 85 kDa epithelial cell receptor (CSL-R).	Riggs et al. (1997), Boulter-Bitzer et al. (2007)
gp900	Adhesion	Highly glycosylated micronemal protein; Has a putative transmembrane domain; Shed from sporozoite surface.	Petersen et al. (1992), Barnes et al. (1998)
gp40/15	Adhesion	Precursor (gp60) is proteolytically cleaved; Both shed during gliding motility; gp15 has a GPI-anchor; gp40 is the soluble N-terminal fragment.	Cevallos et al. (2000a, b), Strong et al. (2000), Wanyiri et al. (2007)
P23	Adhesion; motility	23 kDa immunodominant protein; Highly conserved; Shed during gliding motility.	Arrowood et al. (1991), Perryman et al. (1996)
P30	Adhesion	Secreted lectin protein; Associates with gp900 and gp40.	Bhat et al. (2007)
TRAP-C1	Adhesion; motility	Transmembrane micronemal adhesin protein;	Spano et al. (1998)
Cp47	Adhesion	Membrane-associated protein; Affinity for epithelial host cell receptor p57.	Nesterenko et al. (1999)
CPS-500	Adhesion; Motility	Polar glycolipid (non-proteinaceous); Secreted by sporozoites during gliding motility.	Riggs et al. (1989)
Cp2	Invasion; Membrane integrity	Localized in the sporozoites and, to a much lesser extent, in parasitophorous vacuole membrane during asexual stage development	O'Hara et al. (2004)
Cpa135	Invasion	~135 kDa protein; Secreted by sporozoites during gliding motility; Found also to be defining the boundary of the parasitophorous vacuole.	Tosini et al. (2004)
CpABC	Transport; Nutrient transport	*C. parvum* ATP-binding cassette transporter; Localized to the feeder organelle.	Perkins et al. (1999)
Hemolysin H4	Membrane lysis	Putative role in disruption of cellular membranes during infection.	Steele et al. (1995)
CpSUB	Invasion	Subtisilin-like serine protease; Involved in the proteolytic cleavage of gp40/15 precursor (gp60).	Wanyiri et al. (2009)
CpMuc1–7	Invasion	CpMuc4 and CpMuc5 are highly polymorphic between *C. parvum*	O'Connor et al. (2009), Paluszynski et al. (2014)

(continued)

Table 4 (continued)

Protein	Putative role(s)	Characteristics	Reference(s)
		and *C. hominis*; CpMuc4 interacts directly with an unknown host cell receptor.	
COWP1–9	Oocyst	Proteins involved in oocyst wall formation.	Templeton et al. (2004)
CP41	Oocyst	~41 kDa oocyst wall protein.	Jenkins et al. (1999)
Hsp70 and Hsp90	Stress tolerance	Heat-shock proteins.	Snelling et al. (2007)
$sPLA_2$	Invasion	Secretory phospholipase.	Pollok et al. (2003)

and transgenics for *C. parvum* promises a better understanding of *Cryptosporidium* biology and the identification and characterization of new drug targets.

5.3 Metabolomics

The metabolomics field pertains the study of all intracellular and extracellular low molecular weight intermediates and end-products of enzyme-catalysed reactions (termed metabolites) within a living system, at a specific time and state, with aid of different analytical techniques (Hollywood et al. 2006; Baidoo 2019; Jadhav et al. 2019). As metabolites play a key role within a cell in response to biological and environmental stimuli, they exhibit great potential as a tool to bridge the knowledge between genotype and phenotype (Schrimpe-Rutledge et al. 2016; Baidoo 2019). Metabolomics offers various applications in health and disease research, which includes the *Cryptosporidium* field, by presenting an opportunity to discover clinical biomarkers, new drug targets and improve diagnostic techniques (Kaddurah-Daouk et al. 2008; Nalbantoglu 2019).

Previous studies at genomic level hypothesized that *Cryptosporidium* is highly dependent on the host ability to provide nutrients for its own survival due to the absence of crucial metabolic pathways for the *de novo* synthesis of cell building blocks such as amino acids, lipids and nucleosides (Abrahamsen et al. 2004; Xu et al. 2004). Currently, only three peer-reviewed research papers have delved into the impact of metabolic changes in hosts infected with *Cryptosporidium* as opposed to non-infected hosts (Ng et al. 2012; Hublin et al. 2013; Miller et al. 2019). All studies emphasized a noticeable change in metabolic activity according to their status of infection. The first study was conducted in human stool with changes in amino acid, nitrogen and carbohydrate metabolism reported after parasite infection, which was credited to a disturbance of the intestinal epithelium permeability in the host (Ng et al. 2012). A parallel study conducted with mice stool samples also described a change in the metabolome profile due to an impairment in intestinal permeability in

the host after infection (Hublin et al. 2013). More recently, a combined in vitro and in vivo study identified numerous biosynthetic pathways to be likely affected due to *Cryptosporidium* infection (Miller et al. 2019).

Finally, a research performed to assess the metabolic changes of healthy and diarrheic calves after infection with different types of pathogens, which included parasites, also found an imbalance of various metabolites present in important metabolic pathways after infection with an etiologic agent (Huang et al. 2020). Whilst there is some published data on the prevalence of different metabolites during *Cryptosporidium* infection, a thorough investigation using a combination of all 'omics approaches are still needed.

6 The Status-Quo in *Cryptosporidium* Research

There have been several advances in *Cryptosporidium* research over the last years, mainly focused on culturing the parasite in vitro, attempts to genetically manipulate its genome, but also towards discovery new compounds against this parasite. These developments, some of which were breakthroughs, provide a stepping stone into our understanding of *Cryptosporidium* biology. This section will be focused on presenting some of these (latest) discoveries.

6.1 In Vitro Culturing of Cryptosporidium

In 1983, Current and Long used endoderm cells of the chorioallantoic membrane (CAM) of chicken embryos to successfully develop the first culture system for *Cryptosporidium* (Current and Long 1983). Albeit the entire development of human and calf isolates of *Cryptosporidium* was observed in CAM, the yield of oocysts recovered was very low (Current and Long 1983). Of the many cell lines tested for in vitro cultivation and propagation of *Cryptosporidium* throughout the years, some have been shown to support the complete development of this parasite, including oocyst production (Table 5). However, despite the efforts of many researchers, in most of the cell lines tested *Cryptosporidium* peak infection occurs after 3–5 days post-infection, at which point *Cryptosporidium*'s growth gradually declines making long-term infection unsustainable (Hijjawi 2003; Thompson et al. 2005). Furthermore, the yield of oocysts produced is low. In fact, the lack of thin-walled oocyst production in vitro has been pinpointed as one of the reasons why most cell lines are unable to maintain *Cryptosporidium* infection long-term, as these oocysts are required for auto-reinfection (Current and Garcia 1991).

Two cell lines have stood out in recent years: the human ileocaecal adenocarcinoma cell line (HCT-8) and the human oesophageal squamous-cell carcinoma cell line (COLO-680N). HCT-8 is currently the gold standard used for study of *Cryptosporidium* biology and has been shown to support the development of *C. parvum*

Table 5 Summary of cell lines reported to support the development of *Cryptosporidium* spp.

Cell line	Cell line description	Oocyst source(s)	*Cryptosporidium* life cycle stage			References
			Asexual	Sexual	Oocysts	
Avian embryos (CAM)	Endoderm cells of the chorioallantoic membrane of chicken embryos	Human; Human isolate passaged in goat; Bovine	Yes	Yes	Yes	Current and Long (1983)
HFL	Human foetal lung cells	Human; Human isolate passaged in goat	Yes	Yes	Yes	Current and Haynes (1984)
PCK	Primary chicken kidney cells		Yes	Yes	Yes	
PK-10	Porcine kidney cells		Yes	Yes	Yes	
HCT-8	Human ileocaecal adenocarcinoma cells	Bovine (*C. parvum* and *C. andersoni*); Bovine isolate passaged in mice; Human	Yes	Yes	Yes	Upton et al. (1994a), Hijjawi et al. (2001), Morgan-Ryan et al. (2002)
MDCK	Madin-Darby Canine Kidney	Bovine	Yes	Yes	Yes	Rosales et al. (1993)
MDBK	Madin-Darby Bovine Kidney	Bovine	Yes	Yes	Yes	Upton et al. (1994a, b), Villacorta et al. (1996)
Caco-2	Human colonic adenocarcinoma cells	Bovine; Lamb; Human	Yes	Yes	Yes	Buraud et al. (1991)
HT29.74	Human enterocyte cell line	Human	Yes	NR	NR	Flanigan et al. (1991)
HEL	Human embryonic lung	Bovine	Yes	Yes	NR	Woodmansee (1986)
HRT-18	Human rectal tumor cells	Bovine	Yes	NR	NR	Woodmansee (1986)
L929	Mouse fibroblast cell line	Bovine; Human	Yes	Yes	NR	McDonald et al. (1990)
RL95–2	Human endometrial carcinoma cell line	Bovine	Yes	Yes	Yes	Rasmussen et al. (1993)
T84	Human colon carcinoma cells	Bovine	Yes	Yes	NR	Adams et al. (1994)
VELI	Derived from primary culture of rabbit auricular chondrocytes	Bovine passaged in lamb	Yes	Yes	Yes	Lacharme et al. (2004)
Peritoneal macrophages	Mouse peritoneal macrophages	Bovine	Yes	Yes	Yes	Martinez et al. (1992)

COLO-680N	Human oesophageal squamous-cell carcinoma	Bovine; Red Deer	Yes	Yes	Yes	Miller et al. (2018), Jossé et al. (2019)
FHs 74 Int	Human non-carcinoma small intestine cell line	Bovine passaged in mice	Yes	Yes	NR	Varughese et al. (2014)

NR not reported

(Hijjawi et al. 2001; Thompson et al. 2005; Hijjawi 2010), *C. hominis* (Hijjawi et al. 2001; Thompson et al. 2005; Hijjawi 2010), and *C. andersoni* (Hijjawi et al. 2002; Thompson et al. 2005; Hijjawi 2010). From a panel of 12 cell lines, Upton and colleagues demonstrated that HCT-8 cells support a higher rate of infection for *C. parvum* than the other cell lines, including MDBK, MDCK, and Caco-2 (Upton et al. 1994a). Hijjawi et al. (2001) was able to maintain parasite infection in vitro for up to 25 days by sub-culturing the infected HCT-8 cell line (Hijjawi et al. 2001). However, as many other cell lines, HCT-8 cell cultures are unable to maintain a sustained infection without sub-culturing, likely due to the low production of infective oocysts. In contrast, COLO-680N maintained viability without sub-culturing and was shown to produce *C. parvum* oocysts for up to 8 weeks (Miller et al. 2018), which were used to successfully re-infect new cell cultures (Miller et al. 2018; Jossé et al. 2019). The work of Miller et al. (2018) provides a promising long-term culture system for *Cryptosporidium* infection at a laboratory scale that does not require specialized equipment or expertise, and a prospect for abolishing the dependency on animals for the propagation of this parasite (Bones et al. 2019).

Whilst each cell line has their own particular advantages and disadvantages, there is one particular limitation HCT-8 and COLO-680N share: they are both carcinoma-derived/transformed cell lines. Transformed cell lines are immortal and their growth can be maintained easily and indefinitely in vitro, but their very nature, however, means that their proteomic profile and morphology can be very different from the non-transformed cells found in vivo. Using primary human intestinal epithelial cells (PECs), Castellanos-Gonzalez et al. (2013) developed a culture system capable of maintaining *Cryptosporidium* infection for 5 days, in contrast to the 2 days attained with HCT-8 cells. In 2019, Wilke et al. (2019) published a report in which they show that stem-cell derived intestinal epithelial cells grown under liquid-air interface (ALI) maintained *C. parvum* infection for at least 20 days, with production of viable and infective oocysts starting at three days post-infection. Primary cells could offer an alternative and more accurate in vitro model to study *Cryptosporidium* infection as they retain tissue markers and offer a more accurate reflection of conditions in vivo. Nevertheless, there are limitations: (1) primary cells are isolated directly from tissues, thus requiring a constant supply from donors; (2) existence of variability among donors; (3) unlike immortalized cell lines such as HCT-8 and COLO-680N, primary cells have a finite lifespan and can only be passaged a few times in vitro before losing viability; (4) despite being cheaper than animal models, isolation and culture of primary cells can still be prohibitive to many research labs; and (5) isolation of primary cells from humans and animals requires ethical approval, which can be time consuming.

6.1.1 Three-Dimensional Culturing

Another parameter to consider when studying *Cryptosporidium* infection, or other intestinal parasites for that matter, is the dimensionality of the cell culture system. Two-dimensional (2D) culture systems are well-established, inexpensive, easily

handled, do not require specialized equipment or expertise, and can easily be adapted to different experimental settings (e.g. drug testing or imaging). However, 2D culture systems do not provide an accurate representation of the complex microenvironment encountered by *Cryptosporidium* during in vivo infection. In contrast, three-dimensional (3D) and organoid-like culture systems can be developed to more accurately simulate the intestinal environment. However, 3D culture systems are less amenable and cannot be easily adapted to different experimental settings, their complexity often makes them difficult to replicate, they require specialized equipment, and are more costly than the traditional 2D culture system. Despite this, several researchers have developed different 3D or organoid-like culture systems for the study and long-term propagation of *Cryptosporidium*. Early efforts were devised by Alcantara Warren et al. (2008) in which HCT-8 cells were grown in a reduced-gravity, low-shear, rotating wall vessel which allowed the development of an HCT-8 organoid-like model closely mimicking the intestinal epithelium. Infection of this culture system with *C. parvum* oocysts resulted in increased growth of the parasite for 48 h, at which point a decline in the intensity of the infection was observed, followed by sloughing (Alcantara Warren et al. 2008). In 2016, a culture system employing HCT-8 cells and hollow fibre technology allowed for a sustained *Cryptosporidium* infection for up to six months, with the production of an average of 10^8 oocysts/mL per day, 100× more than the equivalent 2D HCT-8 culture system (Morada et al. 2016). The hollow fibre technology allows for a controlled biphasic environment which provided the authors with the opportunity to supply the parasite with a specifically formulated medium, whilst maintaining a separate environment and medium for host cell maintenance. The culture system developed by Morada et al. (2016) provides a long-term system for the propagation of *C. parvum*, with high yields of oocysts which are able to infect immunocompromised mice. However, this culture system is not amenable to study host-parasite interactions, test different time points and/or conditions, or drug-screening, as it is difficult to manipulate (Karanis 2018; Bones et al. 2019). More recently, DeCicco RePass et al. (2017) described the development of a 3D intestinal model using a silk fibre scaffold seeded with Caco-2 and HT29-MT cells. This culture model supported *C. parvum* infection for up to 15 days with oocyst production and permitted the transfer of *C. parvum*-infected cells from one scaffold to a new one, albeit the authors only performed a total of three of such passages in their work. Unlike the hollow fibre system, the silk scaffold system is better suited for host-parasite interaction studies, as well as drug screening on the account of its smaller scale (DeCicco RePass et al. 2017; Karanis 2018). In contrast, the oocyst yields are lower than those produced with hollow fibre making the silk scaffold system unsuitable for large-scale propagation by comparison (DeCicco RePass et al. 2017). A disadvantage common to all three 3D culture systems described above is that they all employ cancer-derived cells, voiding one of the advantages that 3D culture systems are supposed to deliver: a more accurate in vitro model of the in vivo environment. Heo et al. (2018) evaded this issue by using organoids derived from human intestinal and lung epithelium. These organoids supported complete development and propagation of *C. parvum* for 28 days, followed by a gradual decrease in parasite growth over time; organoid-

produced oocysts were low in number, but still infectious to mice. Organoid systems can be grown as 2D cultures as well, providing this system with a layer of flexibility not offered by the other 3D culture systems mentioned herein. Nevertheless, whilst it provides a very good alternative to the transformed cells-based 3D systems, the model described by Heo et al. (2018) has its limitations: (1) organoids are derived from human tissues, which implicates ethical approval as well as introduces variability due to different donors; and (2) infection of individual organoids was achieved by microinjection of *C. parvum* oocysts, thus it is not easily scalable or manipulated.

Ultimately, the study of *Cryptosporidium* biology and infection is still hampered by the lack of a standard culture system that sustains long-term culturing and propagation of this parasite. Many cell-lines and cell culturing systems have been described so far, but careful consideration needs to be taken by researchers when choosing which to use in their experimental setting.

6.2 *Genetic Manipulation of* Cryptosporidium

The study of *Cryptosporidium* biology has been disadvantaged not only due to lack of efficient and standardized culture system, to allow the long-term propagation of *Cryptosporidium*, but also due to absence of molecular tools which allow for the genetic manipulation of this organism.

The first step toward the establishment of gene editing tools for *Cryptosporidium* was taken by Vinayak et al. (2015). In their work, they started by transfecting *C. parvum* sporozoites with a plasmid containing the translational fusion of the nanoluciferase (Nluc) reporter gene and the neomycin resistance marker (NeoR), flanked by *C. parvum* regulatory sequences (Vinayak et al. 2015). Transient transfection was successfully obtained in vitro after infection of HCT-8 cell cultures with the transgenic sporozoites. However, as previously mentioned, there is currently no in vitro system which allows for the long-term propagation of *Cryptosporidium*. To circumvent this issue, Vinayak et al. (2015) developed a surgical procedure that allowed the direct delivery of sporozoites into the small intestine of susceptible, interferon-γ knockout mice. Thick-walled oocysts excreted in the feces of infected mice were purified and used to successfully infect HCT-8 cell cultures and immunodeficient mice. After establishing a robust system that allows the engineering and propagation of transgenic lines of *C. parvum*, the efforts of Vinayak and colleagues focused on developing a CRISPR/Cas9-based system (reviewed in Doudna and Charpentier 2014 and Lino et al. 2018) to engineer a stable gene knockout strain of *C. parvum*. Sporozoites were transfected with a plasmid containing a gene for a single guide RNA (sgRNA) to target thymidine kinase (TK)—an enzyme involved in pyrimidine metabolism and hypothesized to be responsible for *Cryptosporidium*'s resistance to anti-folates—, and the Cas9 endonuclease gene from *Streptococcus pyogenes*. Successful targeted deletion of TK gene resulted in a strain of *C. parvum* more sensitive to trimethoprim than the wild-type strain, thus showing that TK is a

non-essential enzyme and its presence limits the efficacy of anti-folate drugs by providing an alternative pathway for synthesis of deoxythymidine monophosphate (dTMP) (Vinayak et al. 2015). The development of this tool opens open up an array of possibilities, such as the construction of attenuated parasites for vaccine development, target-based study for drug development, and phenotypic screening in vitro and in vivo. However, it comes with some limitations: (1) it is a laborious and expensive method; (2) it relies on infection of immunodeficient mice for propagation of the transgenic parasite lines, a resource that not all research laboratories have at their disposal; (3) there is the potential for off-target effects, a problem inherent to the CRISPR/Cas9 system; (4) ablation of essential genes is not possible; and (5) this system is not compatible with the scale-up required for drug screening (Beverley 2015; Bhalchandra et al. 2018; Lino et al. 2018).

6.2.1 Gene Silencing Using RNA Interference

Whilst the development of a CRISPR/Cas9-based system in *Cryptosporidium* is in itself promising and a major accomplishment, it is important to develop different methods for the genetic manipulation of this parasite, especially methods that can address, or circumvent, some of the limitations of the CRISPR/Cas9-based system. Gene silencing using RNA interference (iRNA) (reviewed in Bantounas et al. 2004, Geley and Müller 2004, and Doench and Novina 2006) has been widely used to study gene function by knocking-down gene expression at a post-transcriptional level. However, *Cryptosporidium* lacks the genes that encode the machinery of the iRNA pathway (Abrahamsen et al. 2004; Xu et al. 2004). To circumvent this issue, Castellanos-Gonzalez et al. (2016) used protein transfection methods to directly introduce a RNA-induced silencing complex (RISC) comprised of a single-stranded RNA (ssRNA)—which guides the complex to the mRNA target via base complementarity—pre-loaded into the human Argonaute 2 (hAgo2) enzyme—which cleaves the targeted mRNA—into *C. parvum* oocysts (Castellanos-Gonzalez et al. 2016). The targeting of different *C. parvum* genes for silencing resulted in the reduction of transcripts for those genes and allowed the study of their role in *C. parvum* development (Castellanos-Gonzalez et al. 2016, 2019), showing this method can be successfully used for genetic manipulation of *C. parvum*. In contrast to the CRISPR/Cas9- based system developed by Vinayak et al. (2015), the gene silencing method described by Castellanos-Gonzalez et al. (2016) provides one main advantage: it is a simpler and faster method. By transfecting oocysts directly with the pre-assembled ssRNA-hAgo2 protein complex the authors essentially fast-track the gene silencing process by bypassing the transcription-translation processes, meaning the parasite is ready to be assayed/analyzed much faster (Castellanos-Gonzalez et al. 2016; 2019; Castellanos-Gonzalez 2020). Moreover, direct transfection of oocysts circumvents the need for in vivo propagation and increases the rate of success, as sporozoites are much more fragile than oocysts and transfection methods can affect sporozoite viability (Castellanos-Gonzalez et al. 2016, 2019; Bhalchandra et al.

2018; Castellanos-Gonzalez 2020). However, limitations of this method include its transient nature, possibility of incomplete protein knock-down, and off-target effects (Geley and Müller 2004; Doench and Novina 2006).

In a more recent study, another gene silencing tool was developed for *C. parvum* using morpholinos antisense oligonucleotides, which are synthetic DNA analogues that bind to the target mRNA by base complementarity and inhibit protein translation initiation (reviewed in Eisen and Smith 2008). Because they easily cross cellular membranes, morpholinos were introduced into sporozoites without the need to use transfection reagents or methods that could be harmful to this fragile form of the parasite, and knock-down expression of selected genes was successful both in vitro (Witola et al. 2017) and in vivo (Zhang et al. 2018). The ability of morpholinos to readily cross cellular membranes, their stability and water-solubility, provide an advantage over iRNA-based tools, as they are more likely to maintain a sustained knock-down for longer periods. However, similar to the iRNA-based method, the gene silencing effect of morpholinos is temporary, there is a possibility for off-target effects, as well as for incomplete protein knock-down (Eisen and Smith 2008).

Despite the promising success of all the new tools for the genetic manipulation of *Cryptosporidium* summarized herein, the CRISPR/Cas9-based system is, perhaps, the most promising as it is currently the only method which allows for complete ablation of the target-gene. The dependency of immunodeficient mice for propagation is a serious limitation for the widespread use of this tool on a basic research setting, one that can hopefully be overcome when a suitable in vitro system for the continuous culture of *Cryptosporidium* is established.

6.3 *Anti-*Cryptosporidium *Drug Development*

Treatment of cryptosporidiosis is currently sub-par, with only one drug—Nitazoxanide (NTZ)—currently being FDA-approved for this specific purpose. However, this drug has been shown to be dependent on a healthy immune system, showing poor efficacy in immunocompromised individuals (e.g. HIV patients) affected with cryptosporidiosis (Abubakar et al. 2007). The current problem concerning the absence of an effective cryptosporidiosis treatment is compounded by obstacles inherent to the study of *Cryptosporidium* and the drug-development process: (1) the lack of widely used and accepted in vitro culture system which allows for the propagation of *Cryptosporidium* long-term (see Sect. 6.1) has limited the understanding of *Cryptosporidium* biology and its interaction with the host, as well as delayed the establishment of standard protocols and tools for the genetic manipulation of this parasite, a necessary step for target-based drug development approaches; (2) the high cost of developing a novel drug, in particular when the target-pathogen for this new drug has a much higher incidence in poorer countries. As such, efforts have been allocated to drug repurposing, i.e. use of existing drugs and drug-like compounds.

Besoff and colleagues conducted the first cell-based high-throughput screening to identify anti-*Cryptosporidium* drugs using *C. parvum*-infected HCT-8 cells (Bessoff et al. 2013). In this study, the NIH clinical collections (NCC) libraries, comprised of 727 FDA-approved drugs, and thus known to be safe in humans and with well-described mechanisms of action, was screened and 16 *Cryptosporidium* growth inhibitors were identified by measuring *C. parvum* growth inhibition with the help of automated image capture and analysis (Bessoff et al. 2013). In a follow-up study Bessoff and colleagues set out again to replicate it, this time by querying Medicines for Malaria Venture (MMV) Open Access Malaria Box, a collection of 400 commercially available compounds which have been shown to exhibit activity against the erythrocytic stage of *P. falciparum*, another Apicomplexan parasite; the screen identified quinoline-8-ol and allopurinol scaffolds to have potent inhibitory activity against *C. parvum* (Bessoff et al. 2014). The observation that the benzimidazole nucleus is at the core of several antiparasitic, antifungal, anthelmintic, and anti-inflammatory drugs prompted Graczyk et al. (2011) to investigate the effects of 11 benzimidazole derivatives on *C. parvum*-infected HCT-8 cells. Nine out of the 11 compounds tested exhibited efficacy against *C. parvum*, and three of the compounds showed superior efficacy when compared to the control paromomycin, a drug commonly used to treat *Cryptosporidium*-infected animals and in in vitro research. In a more recent study Love et al. (2017) screened a total of 78,942 compounds for anti-cryptosporidial activity. Whilst a total of 12 compounds exhibited potent activity against both *C. parvum* and *C. hominis*, only Clofazimine demonstrated a good therapeutic index, meaning that there was no toxicity or undesirable effects on the HCT-8 host cell line (Love et al. 2017). Further testing of Clofazimine demonstrated that this drug also dramatically reduced oocyst shedding in mice after only one single oral dose (Love et al. 2017), establishing Clofazimine as a potential drug for the treatment of cryptosporidiosis.

Whilst cell-based high-throughput screening studies offer the advantage of rapidly screening hundreds, if not thousands, of compounds, they also have limitations: large-scale drug testing is expensive and requires specialized liquid-handling robots to minimize errors and provide a faster set-up, something which is often outside the capability of a typical research laboratory. Furthermore, cell-based assays often also require additional secondary in vitro screens to assess the pharmacokinetics and pharmacodynamics of the drug.

The availability of the genome sequences of *C. parvum* and *C. hominis* (Abrahamsen et al. 2004; Xu et al. 2004) has provided insight into the biology of this parasite, as well as offer potential new therapeutic targets. Previous observations showing that *Cryptosporidium* exhibited some resistance to drugs which were effective against other related parasites stunned researchers for some time (Woods et al. 1996). However, with sequencing and analysis of *C. parvum* and *C. hominis* genome it became obvious that, unlike other Apicomplexan parasites, *Cryptosporidium* did not contain an apicoplast nor a typical mitochondrion and, as such, classic drugs which target and inhibit the metabolic pathways usually carried out within these organelles (i.e. oxidative phosphorylation, fatty acid oxidation, and TCA cycle) are woefully inadequate, as those pathways are also absent. Further sequence

analysis of the genomes revealed a very streamlined metabolism and a large dependence of *Cryptosporidium* on nutrient scavenging from the host, including amino acids, sugars, and nucleotides (Abrahamsen et al. 2004; Xu et al. 2004). Despite revealing a lack of conventional drug targets, sequence analysis of the *Cryptosporidium* genome also revealed potential alternative drug targets—further analysis showed that this parasite encodes several genes for putative sugar transporters and amino acid transporters (Abrahamsen et al. 2004; Xu et al. 2004). Combined with the dependency of *Cryptosporidium* on nutrient acquisition from the host, these transporters may provide potential targets for drug development. Genome sequence analysis also showed that *Cryptosporidium* has acquired genes from bacteria by horizontal gene transfer (Striepen et al. 2002, 2004; Huang et al. 2004b) providing several new potential targets with activity distinct from homologues found in humans. Among those potential new targets is *Cryptosporidium*'s inosine 5-′-monophosphate dehydrogenase (CpIMPDH). This enzyme appears to be the only pathway which allows *Cryptosporidium* to acquire guanine nucleotides (Striepen et al. 2004; Kirubakaran et al. 2012), and due to its bacterial origin (Striepen et al. 2002, 2004) it exhibits higher similarity with its bacterial homologues than with its human homologues (Striepen et al. 2004; Umejiego et al. 2004; Mandapati et al. 2014).

The availability of genome sequences also provided us with protein sequences and a putative proteome. *In silico* proteomic approaches offer the opportunity to predict the structure and characteristics of those proteins. Taking advantage of the vast array of bioinformatic tools available, Shrivastava et al. (2017) identified 105 hypothetical proteins from both *C. parvum* and *C. hominis* using CryptoDB.org, followed by a BLAST search which identified hypothetical protein TU502HP of *C. hominis* as a unique protein. Further *in silico* analysis was able to identify a putative inhibitor for this protein. Whilst *in silico* analysis of genome and protein sequences is in itself a powerful tool, it is important to note that any potential target and drugs identified using this approach will always require in vitro and in vivo validation, both of which, in the case of *Cryptosporidium*, presently still lack proper standardization.

7 Conclusion

In this book chapter, we provided the basic information and the current status quo regarding the biology of *Cryptosporidium*, focusing in taxonomy, prevalence and zoonotic potential, morphology and life cycle, but also providing a summary of the most recent accomplishments on understanding the pathogenicity of the parasites, and methods to eradicate them. One important issue that was not assessed is the relationship between *Cryptosporidium* and the host microbiome. Currently, only a handful of reports exist that have investigated the composition of the host gut microbiome prior to, or during, *Cryptosporidium* infection. For example, one report has investigated the effect of probiotics on susceptibility of mice to cryptosporidiosis

(Oliveira and Widmer 2018), whilst others have focused on the composition of certain prokaryotic communities during *Cryptosporidium parvum* infection in neonatal calves (Ichikawa-Seki et al. 2019). At present, only one survey exists exploring the effect of the gut microbiome during *Cryptosporidium* infection, where it was assumed that the parasite depletes the gut microbiome in Coquerel's sifakas (McKenney et al. 2017). Currently, reports presenting the overall effect of *Cryptosporidium* parasites in the human gut microbiome are lacking, whilst descriptions demonstrating the influence of the gut microbiome to the parasite infection are non-existent. Further investigations on this subject would provide us with better understanding on the biology, host-specificity and host-parasite interactions and assist the scientific community in developing new and efficient interventions to combat this parasite.

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