Chapter

State of the Art and Future Directions of *Cryptosporidium* spp.

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Abstract

Cryptosporidium species are protozoan parasites that infect epithelium surfaces in gastrointestinal and respiratory tracts of humans and a range of animals worldwide. Cryptosporidiosis has been associated with considerable morbidity and, under certain circumstances, mortality. Humans can acquire it by consuming food and drink containing oocysts, which have been recognised as a major cause for diarrhoeal disease. The ubiquitousness of the infective oocyst, its resilience to environmental pressures, and the low dose of oocyst exposure needed for infection amplify to outbreaks of *Cryptosporidium* traced to drinking and recreational water. Unlike in developing countries where lack of sustained access to safe water creates tremendous burdens of *Cryptosporidium* diarrhoea, this scenario is aggravated due to limited diagnosis and therapeutics. However, over the past few decades, growing information on *Cryptosporidium* genomes have allowed novel insight into the host-parasite relationship. Future field research on potential tools will focus on biology-derived parasite products applicable to drugs and diagnosis. This chapter reviews available data on biology, transmission, life cycle, diagnosis, genome, and a few but important progresses in the field of cryptosporidiosis.

Keywords: cryptosporidiosis, diagnosis, transmission, infectious disease, genome

1. Introduction

Cryptosporidium species are protozoan parasites that infect the epithelial cells of the gastrointestinal and respiratory tracts of humans and a wide range of animals, with a global distribution [1–3]. *Cryptosporidium* represents a major public health concern for waterborne disease and daycare outbreaks of diarrhoeal disease worldwide [2, 4–8]. Human cryptosporidiosis is usually a self-limiting infection in immunocompetent individuals. However, cases of severe diarrhoea and dissemination to extra-intestinal sites can occur in children, the elderly, and individuals with impairment of T-cell functions, mainly those with HIV infection [9–12]. In children, although diarrhoea is a key feature of malabsorption, it may not be apparent at presentation; when the infection becomes chronic, the only symptom may be limited growth. Consequently, chronic infections can culminate in poor growth [5, 13–16]. The epidemiology of infections is complex and involves transmission by a faecal-oral route, either by ingestion of contaminated water or food or by human-to-human or animal-to-human transmission [17, 18]. The oocyst, the environmental stage of *Cryptosporidium,* is incredibly hardy, easily spread through water, and resistant

to inactivation by chlorine; and without the use of filtration, it is challenging to remove it from drinking water [19–21]. *Cryptosporidium* prevalence is higher in areas lacking a sanitation infrastructure, mainly drinking water and sewage, which led the World Health Organization (WHO) to include it in the water sanitation and health programme [22]. The scarcity of sustained access to safe water creates tremendous burdens of *Cryptosporidium* diarrhoea in developing countries [23]. Treatment and diagnosis options are still not totally effective [2, 24–26]. No fully effective drug therapy or vaccine is available for *Cryptosporidium*, and the diagnosis of cryptosporidiosis has been based on the demonstration of oocysts in faeces, which present low sensibility [25]. However, the ability to culture relevant *Cryptosporidium* isolates in vitro, the development of novel gene-editing tools (knockout genes, CRISPR/Cas9, and RNAi) [26–30], and 'omic' research (genomics, transcriptomics, and proteomics) represent essential paths towards significant advancements in the control of cryptosporidiosis [30–38]. In the future, those approaches will show a holistic view of the biology of *Cryptosporidium*. In this chapter, we present recent advances and remaining challenges regarding human cryptosporidiosis under a public health perspective.

2. Clinical perspective, diagnosis, and treatment

Despite *Cryptosporidium* species infecting the epithelial cells of the gastrointestinal and respiratory epithelium tracts, human cryptosporidiosis is a usually self-limiting infection in immunocompetent individuals with a low fatality rate [39–41]. In general, onset of the symptoms occurs 5–7 days following exposure and resolves in 2–3 weeks [42]. Clinical manifestations vary from subclinical infection to watery diarrhoea, sometimes profuse. Other common symptoms include abdominal cramps, fever, flatulence, nausea, vomiting, and low-grade fever [43–45]. Clinical presentation of cryptosporidiosis in individuals with impairment of T-cell functions, mainly those with HIV infection, varies according to the level of immunosuppression, from asymptomatic disease, to transient disease, to relapsing chronic diarrhoea or even cholera-like diarrhoea that is debilitating and potentially life-threatening [46]. Spreading of infection beyond the extra-intestinal site (in the biliary or respiratory tract) has been documented in children and immunocompromised people, resulting in a potentially life-threatening disease [47, 48]. Sclerosing cholangitis and other biliary involvements are common in AIDS patients with cryptosporidiosis. Both innate and adaptive immunity of the host have major impacts on the severity of cryptosporidiosis and its prognosis.

Cryptosporidium has been diagnosed using a variety of approaches, such as microscopy, immunofluorescent antibody (IFA), enzyme-linked immunosorbent assay (ELISA), and DNA-based detection methods [18]. However, identification of the parasite's morphologic features through examination of stool smears is widely employed in diagnostic laboratories, particularly in resource-limited health systems. The oocysts are shed intermittently [49]; therefore, three faecal samples collected on alternate days are recommended. To maximise the recovery of oocysts, Sheather's sucrose flotation, saturated salt flotation, and Allen and Ridley's formol-ether method are the stool concentration techniques most frequently used prior to the use of the microscopy staining technique [50, 51]. Stain differential is required due to the small size of the specimen (ranging from 4 to 6 μm), similar in shape to yeasts and faecal debris [52]. Safranin-methylene blue, Kinyoun Ziehl-Neelsen, and dimethyl sulfoxide-carbol fuchsin are the most commonly used stain methods [11, 53–55]. However, in the absence of staining solution, phase contrast microscopy has proven to be highly specific for the detection of *Cryptosporidium* oocysts in human stool

samples [56]. In general, conventional microscopy lacks sensitivity, is time-consuming, and requires a skilled and well-experienced microscopist [57–59].

Direct fluorescent antibody tests (DFAs), enzyme-immunoassays (EIAs), and rapid immunochromatographic assays (dipsticks) are commercially available [60–63]. The EIA kits have been evaluated with human stool specimens only, presumably from patients infected with *C. hominis* or *C. parvum*. The direct fluorescent antibody tests have been widely used for the detection of *Cryptosporidium* in faecal smears, water, and food [60, 62–66]. However, the antigenic variability of oocyst wall epitopes contributes to reducing specificity, and the sensibility of all immunological-based methods is low. High specificity (99–100%) has been generally reported for EIA kits. Sensitivities, however, have been reported to range from 70 to 100% [62–65]. Dipsticks and EIAs are available for individual and for all-in-one tests for *Giardia*, *Cryptosporidium*, and *Entamoeba histolytica* [66–69]. The tests are fast and easy to perform. However, EIA kits and rapid format assays present a potential problem with false positives, so results need to be interpreted and evaluated with caution [70]. To overcome these barriers, one of the most notable advances in public health in recent decades has been the development of tools based on molecular biology for the diagnosis of infectious diseases. These polymerase chain reaction (PCR) techniques have enabled specific sensitive detection of oocysts (a single oocyst) in clinical and environmental samples [71–77]. Examples of such techniques include conventional PCR, quantitative PCR real time, and high-resolution melt. A wide variety of PCR methods targeting different genes have been developed for the detection of *Cryptosporidium* at the species/genotype/subtype levels. However, no targeted tests have been patterned for the detection of *Cryptosporidium* in clinical laboratories. Recently, the simultaneous qualitative detection and identification of multiple viral, parasitic (including *C. parvum* and *C. hominis*), and bacterial nucleic acids in human stool specimens were approved by the Food and Drug Administration (FDA) [78]. In general, PCR tools solely amplify the DNA of *C. parvum*, *C. hominis*, *C. meleagridis*, and species/genotypes closely related to *C. parvum* [18]. For genotyping, nested PCR-RFLP was the most commonly used method in the past. Nowadays, DNA sequencing of 18S has been required to reliably detect all *Cryptosporidium* spp. The HSP70 and COWP targets fail to detect the DNA of *C. felis*, *C. canis*, and *C. muris* [79]. Subtyping tools are indispensable from the epidemiological point of view and are helpful in knowing the possible transmission routes of *Cryptosporidium* species and zoonotic potential of the parasite. Several subtyping tools have been developed to evaluate the diversity within *C. parvum* or *C. hominis*, including analysis of the microsatellite, GP-60 gene, HSP70 gene, 47-kDa protein, small double-stranded (ds) RNA virus, serine repeat antigen, and T-rich gene fragment [73, 80–85]. The 18S ribosomal RNA (rRNA) gene and the hypervariable 60-kDa glycoprotein (gp60) gene have been widely used as targets to identify species and track transmission [18, 86, 87]. The 60-kDa glycoprotein (gp60, also known as gp40/gp15) gene presents a wide genetic heterogeneity in the number of trinucleotide repeats (TCA, TCG, or TCT). This gene encodes a precursor protein that is cleaved to produce mature cell surface glycoproteins (gp45/gp40 and gp15) implicated in the attachment to, and invasion of, enterocytes [18, 87]. Identification of subtypes using GP60 subtype families has revealed the subtype families (Ia-Ik) in *C. hominis* [87–91] and two zoonotic subtypes (IIa, IId), subtypes (IIb, IIc, IIe, IIf, IIi, IIj-IIt) in *C. parvum* [4, 87, 92–94], and subtype families (IIIa to IIIg) in *C. meleagridis* have been acknowledged [87, 95, 96]. Subtyping tools targeting the gp60 gene have been developed recently for several other human-pathogenic *Cryptosporidium* species [87]. Species and subtype identification are not necessary for clinical care and therapeutic options but are important for epidemiological surveillance and for drug investigations and clinical trials. Novel diagnostic tools and biomarkers for

cryptosporidiosis, which could also be used for therapeutic or vaccine trials, are necessary for accurate identification.

Current treatment options for cryptosporidiosis are limited. So far, there is no vaccine against *Cryptosporidium* [97], and nitazoxanide (NTZ) is the only drug approved by the FDA for treatment of cryptosporidiosis in children and immunocompetent adults [98]. However, it is not effective without an appropriate immune status and, consequently, is ineffectual for the treatment of immune-compromised patients, particularly those with AIDS [25, 99]. NTZ is a nitrothiazole benzamide compound with a broad spectrum of activity against a wide range of parasites, bacteria, and viruses. In protozoa, NTZ inhibits the enzyme pyruvate ferredoxin oxidoreductase, which is essential to anaerobic energy metabolism [100]. Due to the prevalence of cryptosporidiosis, the development of novel therapeutic targets and vaccines against *Cryptosporidium* spp. is a public health priority. The ongoing need to develop new anti-cryptosporidial drugs has spurred the process of finding new uses for existing drugs. Repurposing drug provides an attractive alternative to drug development [101]. Two compounds, 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitor, pitavastatin and auranofin (approved for the treatment of rheumatoid arthritis), have been shown to be effective against *Cryptosporidium* in vitro [102]. Auranofin has been shown to be 10 times more potent than metronidazole against *Entamoeba histolytica*, the protozoan agent of human amoebiasis [103]. HMG-CoA and auranofin have particular promise in fast-tracking for further in vivo testing in animals and humans.

3. Life cycle and classification

The parasite has a complex monoxenous life cycle with both asexual (merogony) and sexual (gametogony) stages. Ingestion of an infective oocyst (containing four sporozoites) by a susceptible host initiates the excystation process in the gastrointestinal tract. The sporulated oocyst ruptures, releasing sporozoites that invade the enterocytes, inducing the cell membrane to enclose the parasite in the parasitophorous vacuole, which then differentiates into a trophozoite. Trophozoites undergo merogony and form either a further type I meront or a type II meront, which contains four merozoites that are destined for gametogony. Merozoites can differentiate into sexually distinct stages called macro- and microgametocytes in a process called gametogony. New oocysts are formed in the epithelial cells from the fusion of a macro- and a microgametocyte to form a diploid zygote. The new fused cell evolves and sporulates in situ in a process called sporogony, becoming oocysts containing four sporozoites. Type II meronts attach to the epithelial cell and differentiate into either macrogamonts or microgamonts. The microgametes from the microgamont are released, and each can fertilised a macrogamont to form a diploid zygote. This cell undergoes a process like meiosis (sporogony) to produce an oocyst, either thin- or thick-walled, containing four sporozoites (sporulated oocysts). The thin-walled oocysts are involved in autoinfection, and thick-walled oocysts are released within the faeces to infect new hosts [104–107] (**Figure 1**).

Until relatively recently, *Cryptosporidium* was classified as a coccidian parasite. However, the taxonomic placement of *Cryptosporidium* was altered after revisions to higher-order classifications due to recent particularities observed in *Cryptosporidium*. The parasite can develop in a cell-free culture, while extracellular stages have been observed in both cell-free and cell cultures, in biofilms, and in vivo [108–111]. It presents the ability to grow and amplify without host cell attachment and encapsulation, as well as the insensitivity of all anticoccidial agents [26]. Moreover, the parasite lacks a micropyle, sporocyst, and polar granular [111–113]. Although initially considered

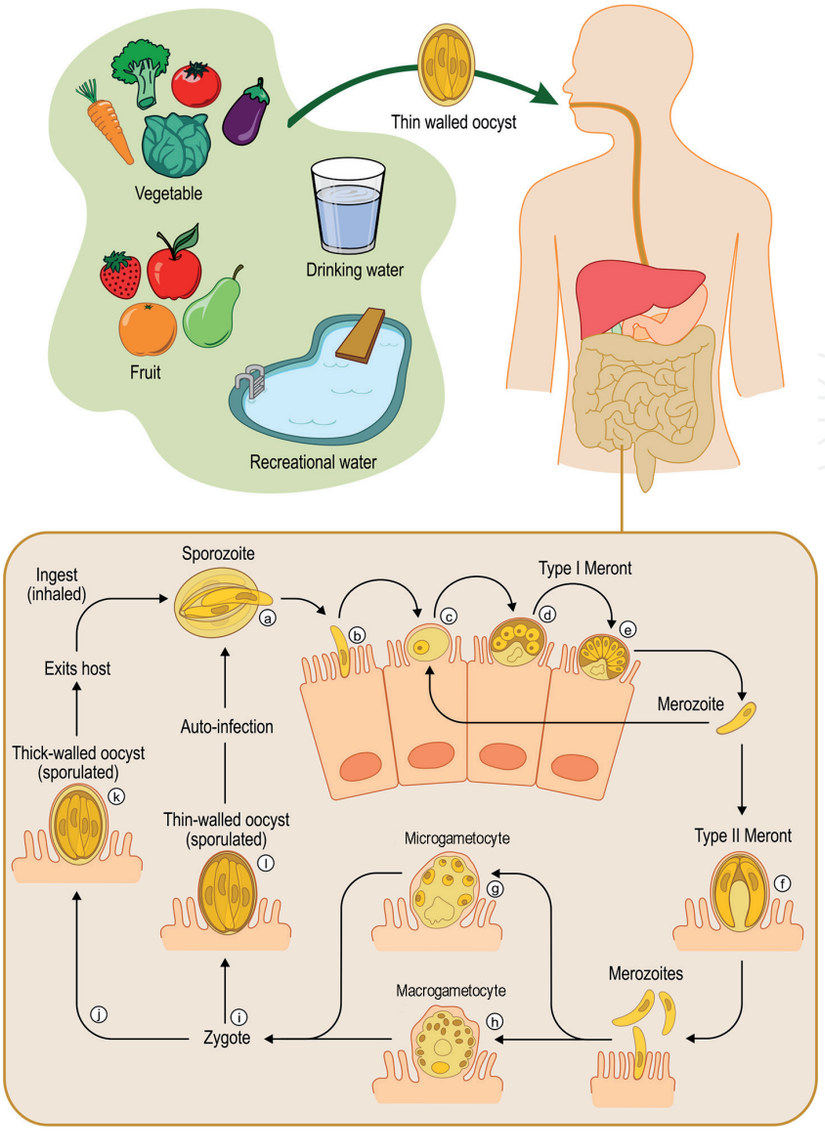

Figure 1.
A schematic diagram of Cryptosporidium *life cycle. After ingestion of contaminated water and/or food, the oocyst wall opens (excystation) triggered by temperature, stomach acid, and bile salts. Then, sporulated oocyst ruptures releasing (a) sporozoites that (b) invade the host cell (c) inducing the cell membrane to enclose the parasite in the parasitophorous vacuole, (d) which then differentiates into a trophozoite that undergoes an asexual reproduction, (e) forming a type I meront that contains 6–8 merozoites. These merozoites can reinfect the epithelial cell, where they undergo merogony and form type I meront or (f) type II meront. (g) Merozoites can differentiate into sexually distinct stages called (g) micro- and (h) macrogametocytes. (i) Zygote is formed after the fertilisation of macrogametocyte by the microgametocyte, (j) and this cell undergoes sporogony and produces a thin-walled oocyst. (k) These thin-walled oocysts are released within faeces to infect new hosts, as well as (l) involved in autoinfection process (adapted from Ref. [104]).*

to be a coccidian, *Cryptosporidium* spp. share features of both the coccidia and gregarines, confirmed by morphological and molecular data. Major similarities between *Cryptosporidium* and gregarine parasites are as follows: (1) the ability to complete its life cycle in the absence of host cells, (2) extracellular gamont-like stages, (3) the process in which two mature trophozoites pair up before the formation of gametocyst (szygy), and (4) changing cell architecture to adapt to diverse environments (biofilms, coelom, intestines, soil, and water) [107, 108, 111, 114]. The most recent classification considers *Cryptosporidium* as a separate group within the Apicomplexa. Analyses of comparative genomics and of phylogenetic inference and the ability of *Cryptosporidium* to complete its life cycle extracellularly confirm its close relationship with gregarines and corroborate the transference of *Cryptosporidium* to the Gregarinomorphea class as a new subclass of Cryptogregaria [111, 115]. Early taxonomy at species level was based originally on morphology and host specificity. Nowadays, the description of species

takes molecular analyses, mainly DNA sequencing and PCR-related methods, into account for the detection and differentiation of *Cryptosporidium* spp.

4. Maintenance of *Cryptosporidium* in nature and transmission

Once excreted into the environment, oocysts can be dispersed from the faecal matrix into the terrestrial environment (**Figure 2**). When present on the soil surface, oocysts may be exposed to high temperatures and desiccation, causing their inactivation. Oocysts are sensitive to desiccation and UV-C irradiation [116]. Reports show that desiccation is lethal to oocysts with only 3 and 5% remaining viable after being air-dried at room temperature for 2 and 4 h, respectively [117, 118]. However, when within the soil column, the oocysts were maintained, protected, and viable [119, 120]. Studies have indicated that oocysts at 4°C recovered from soil column may remain infectious for long periods [119, 121]. These findings suggest that the soil column is a sanctuary for *Cryptosporidium*, protecting it until rainfall events scatter them [120]. Oocysts were able to remain viable and infectious after being frozen at −10°C for up to 168 h, at −15°C for up to 24 h, and at −20°C for up to 8 h [122]. Moreover, *Cryptosporidium* oocysts can be carried in the environment due to interactions with biofilms (surface-attached microbial communities). They readily attach to biofilms and persist and subsequently separate from it. High concentrations of oocysts in water biofilms that were maintained over several months maintained viable sporozoites [123]. *Cryptosporidium* oocysts in fresh water and marine water can survive at a range of temperatures. Fayer et al. reported that oocysts maintained at 20°C remain infectious for 12 weeks at salinities of 0 and 10 ppt, for 4 weeks at 20 ppt, and for 2 weeks at 30 ppt [124]. Although salinity can have a pronounced effect on oocyst infectivity, they can survive long enough in marine waters to justify their presence in marine animals.

Cryptosporidium spp. have a huge impact on both human and veterinary health worldwide, aggravated by the limited diagnosis and current therapeutics. *Cryptosporidium* spp. have a worldwide distribution and the ability to infect a wide range of hosts, including humans, and a broad variety of vertebrate [1, 3]. Humans can acquire cryptosporidiosis through several transmission routes, such as direct contact with infected persons or animals and consumption of contaminated water (drinking or recreational) or food (**Figure 3**).

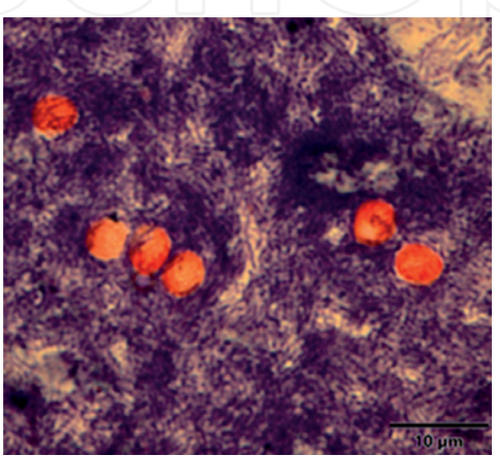

Figure 2.
Cryptosporidium *sp. oocysts in safranin-methylene blue staining method.*

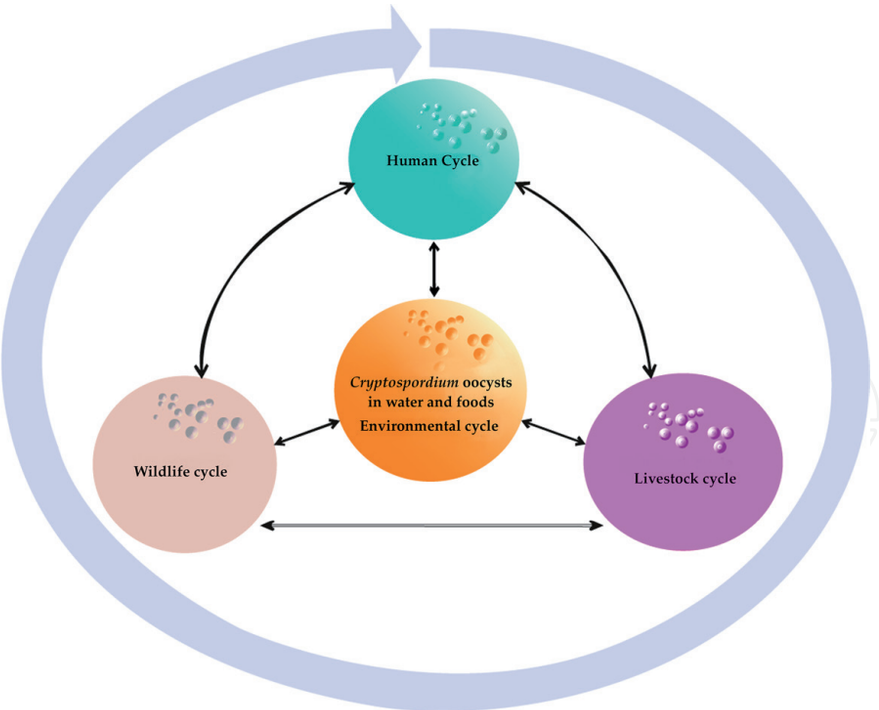

Figure 3.
Transmission cycles of Cryptosporidium *infections.*

The WHO has categorised *Cryptosporidium* as a reference pathogen for the assessment of drinking water quality [125]. Susceptibility to cryptosporidiosis depends on several factors, including environmental conditions, host immune status, age, geographic location, and contact with infected humans/animals [126]. Animals play an important role in the maintenance, amplification, and transmission of *Cryptosporidium* [127]. In fact, a large range of animals are reservoirs for some species, genotypes, and subtypes, which may infect humans [128–130]. The lack of adequate instruments to continuously monitor animal mobility makes it difficult to study the dynamics of transmission [131, 132]. Also, oocysts are ubiquitous in the environment and easily spread via drinking water, recreational water, and food [3, 133, 134]. The ubiquitousness of the infective oocyst, its resilience to environmental pressures [135], and the low-dose oocyst exposure (ingestion of fewer than 10 oocysts can lead to infection) [136, 137] amplify to outbreaks of *Cryptosporidium* traced to drinking and recreational water. In 1993, the largest *Cryptosporidium* waterborne outbreak was recorded in the United States in Milwaukee, where more than 400,000 people were infected by the drinking water supply [138]. The epidemiology of infection is complex and involves transmission by the faecal-oral route, either by indirect transmission through ingestion of contaminated water or food or by direct human-to-human or animal-to-human transmission [3]. The genus *Cryptosporidium* has about 30 species formally described, as well as various genotypes and subtypes. Some species are relatively promiscuous in terms of host specificity, some of which also infected humans. Currently, a wide range of *Cryptosporidium* species and various genotypes have been recognised as responsible for human cryptosporidiosis (**Table 1**).

Human infections predominantly are caused by *C. hominis*, which are considered restricted to humans (anthroponotic transmission), and by *C. parvum*, some of which isolate genotypes and infect ruminants (zoonotic transmission) [18]. However, in recent years, *C. meleagridis*, *C. cuniculus*, and *C. ubiquitum* have also emerged as species relevant to public health, while the other species tend to be associated only with sporadic and rare cases of human infection. Approximately 155

Cryptosporidium spp.	Major host	References
C. andersoni	Cattle	[139–144]
C. baileyi	Chickens and turkeys	[145]
C. bovis	Cattle	[146, 147]
C. canis	Dogs	[148–150]
C. cuniculus	Rabbits	[151–155]
C. fayeri	Marsupials	[141, 156]
C. felis	Cats	[93, 157, 158]
*C. hominis**	Humans	[18]
*C. meleagridis**	Turkeys, chickens, humans	[18, 93, 153, 159, 160]
C. muris	Rodents	[161–163]
*C. parvum**	ruminants, especially calves	[18]
C. scrofarum	Pigs	[164]
C. suis	Pigs	[139, 160, 165–167]
C. tyzzeri	Rodents, snake	[168]
C. ubiquitum	Sheep and cervids	[152, 154, 157, 158, 169]
C. viatorum	Humans	[93, 170]
C. erinacei	Hedgehogs and horses	[171, 172]
C. wrairi	Guinea pigs	[173, 174]
C. xiaoi	Sheep and goat	[174]
Cryptosporidium Chipmunk genotype	Rodents	[93]
Cryptosporidium Horse genotype	Horses	[152, 153]
Cryptosporidium Mink genotype	Minks	[175]
Cryptosporidium Monkey genotype	Monkey	[152]
Cryptosporidium Skunk genotype	Skunk	[152, 153]

**The most prevalent species.*

Table 1.
Currently recognised species of Cryptosporidium *spp. associated with human infections.*

species of mammals have been reported as non-human hosts of *C. parvum*, indicating that the parasite is adapting and developing in many hosts [176].

The human-to-human spread is particularly well described within families (often secondary cases after a primary outbreak infection) in childcare nurseries, nursing homes, and hospitals [42, 177, 178]. In addition, contact with production animals, mainly cattle, that are the main hosts of *C. parvum* can potentially infect humans [40, 178, 179]. To date, studies in developing countries have shown a predominance of *C. hominis* in HIV-positive children and adults. These findings are also valid in the United States, Canada, Australia, and Japan. In Europe and New Zealand, several studies have shown a similar prevalence of *C. parvum* and *C. hominis* in immunocompetent and immunocompromised individuals. Thus, in most developing countries, the anthroponotic transmission of *Cryptosporidium*

plays an important role in human cryptosporidiosis [18, 180], while in Europe, New Zealand, and rural areas of the United States, there are both anthroponotic and zoonotic transmissions. In Middle Eastern countries, children are mainly infected with *C. parvum*, but the significance of this occurrence is not clear [181]. An exception is *Cryptosporidium* infections in HIV-positive patients in Ababa, Ethiopia, where *C. parvum* is highly endemic and where contact with calves is an important risk factor for cryptosporidiosis [174].

In developing countries, most *C. parvum* infections in HIV-positive children and adults are caused by subtype IIc, with IIa largely absent, indicating that anthroponotic transmission of *C. parvum* is common in these areas. Conversely, families of subtype IIa are commonly diagnosed in humans in industrialised regions, where their occurrence is often associated with contact with calves. Another family of *C. parvum* subtypes commonly found in sheep and goats, IId, is dominant in humans in Middle Eastern countries and is occasionally found in humans in some European countries, such as Sweden, where it is commonly diagnosed in dairy calves. A systematic review of the anthroponotic transmission of *Cryptosporidium* concluded that subtype IIc predominates in low-income countries with poor sanitation and in HIV-positive individuals, unlike in higher-income countries, where it is rarely evident. Lacking effective treatment or vaccine, intervention to improve basic sanitation in these regions is the best option. This prophylactic action certainly may reduce the anthropogenic and zoonotic transmission of cryptosporidiosis, reducing the damage to human health. It is important to emphasise the importance of personal hygiene practices to minimise cryptosporidiosis, in addition to other pathogens transmitted by water and food.

5. Genome of *Cryptosporidium*: new insight and future challenges

Recent years have seen impressive progress of next-generation sequencing technologies in genome assembly and annotation methodologies, mainly by advancements in the fields of molecular biology and technical engineering and by reducing cost. *Cryptosporidium* has been the subject of genome sequencing projects, which have provided valuable insights into the species, biology, and host-parasite relationships. The genomic data of multiple *Cryptosporidium* species are available and accessible in a *Cryptosporidium*-dedicated database, CryptoDB (http://cryptodb.org/cryptodb/) [182], and in the GenBank database (www.ncbi.nlm.nih.gov). Comparative analyses have shown that *Cryptosporidium* genomes are highly compact, containing 8.50–9.50 megabase pairs (Mbp), a total gene count ranging from 3769 to 7610, and coding sequence composition (75–77.6%). Moreover, in general, they share a comparable GC percentage (**Table 2**).

Overall, gene content and genomic organisation among intestinal occurrences of the species are well conserved, with *Cryptosporidium* gene clusters encoding putative secreted proteins. Comparison of the *Cryptosporidium* genomes has identified a core set of proteins commonly studied, as well as major differences in particular gene families, which could be involved in biological differences between species and genotypes [114, 183–185]. Gene encoding proteins that are associated with invasion processes, e.g. protein kinases and thrombospondin-related adhesive proteins (TRAPs), insulinase-like peptidases, MEDLE secretory proteins, and mucin glycoproteins, are observed in genome *Cryptosporidium* spp. [32]. However, some of them differ in copy number variations of genes. Comparative genomic analysis revealed that one of the primary features differentiating *Cryptosporidium* species is the sequence diversity present in major secreted protein families, MEDLE, and insulinase-like proteases [184]. This is consistent with transcriptomic studies

Organism/name	Strain	Bio-sample	Bio-project	Size (Mb)	GC %	Gene	Protein
C. hominis	—	SAMEA 3496639	PRJEB 10000	9.10	30.1	3818	3817
C. hominis	TU502	—	PRJNA 13200	8.74	30.9	3949	3885
C. hominis	TU502_2012	SAMN 02382005	PRJNA 222836	9.10	30.1	3796	3745
C. hominis	UKH1	SAMN 02382004	PRJNA 222837	9.15	30.1	3769	3718
C. hominis	30,976	SAMN 02862040	PRJNA 252787	9.06	30.1	3995	3959
C. parvum	Iowa type II	SAMN 02952908	PRJNA 144	9.10	30.2	7774	7610
C. andersoni	30,847	SAMN 04417240	PRJNA 354069	9.09	28.5	3897	3876
C. meleagridis	UKMEL1	SAMN 02666797	PRJNA 222838	8.97	31.0	3806	3753
C. meleagridis	UKMEL4	SAMN 08383028	PRJNA 315503	8.79	30.9	—	—
C. meleagridis	UKMEL3	SAMN 08383027	PRJNA 315502	8.70	31.0	—	—
C. ubiquitum	39,726	SAMN 02768023	PRJNA 534291	8.97	30.8	3766	3766
Cryptosporidium sp.	Chipmunk LX-2015	SAMN 03281121	PRJNA 272389	9.51	31.9	—	—
Cryptosporidium sp.	37,763	SAMN 10623052	PRJNA 511361	9.05	32.0	—	—
*C. baileyi**	TAMU 09Q1	SAMN 02382006	PRJNA 222835	8.50	24.2	—	—
C. cuniculus	UKCU2	SAMN 08383019	PRJNA 3154496	9.18	25.8	—	—
C. muris	RN66	SAMN 02953683	PRJA 19553	9.25	28.5	—	3934
C. viatorum	UKUIA1	SAMN 10107889	PRJA 492837	9.26	31.1	—	—

*Draft genome.

Table 2.
Genomic features of Cryptosporidium *spp.*

of *C. parvum*, which have demonstrated MEDLE proteins in different subcellular locations that may perform their functions in distinct stages of the invasion and development process [33]. Moreover, a reduction in the number of genes encoding secreted MEDLE and insulinase-like proteins was observed in *C. ubiquitum* and *C. andersoni*, whereas the mucin-type glycoproteins are highly divergent between the gastric *C. andersoni* and intestinal *Cryptosporidium* species [184]. Unlike most other apicomplexans, *Cryptosporidium* spp. have no apicoplast or mitochondrial genomes but have remnant ones, the so-called mitosomes. However, *Cryptosporidium* species disagree from each other mostly in mitosome metabolic pathways. *C. parvum*, *C. hominis*, and *C. andersoni* present more aerobic metabolism

and a conventional electron transport chain [114], whereas *C. ubiquitum* has further reductions in ubiquinone and polyisoprenoid biosynthesis and has lost both the conventional and alternative electron transport systems, unlike *C. muris* genome encoding core enzymes for the Krebs cycle and a functional ATP synthase. Thus, the mitosome of *C. muris* functions essentially as a peculiar mitochondrion [186]. However, the loss of biosynthetic pathways is a common feature observed in *Cryptosporidium* spp. genomes, e.g. the cytochrome-based respiratory chain and main de novo synthetic pathways for amino acids, nucleotides, fatty acids, and the Krebs cycle [32, 183]. Conversely, families of transporters to acquire nutrients from the host were expanded, including transporters for amino acids, sugars, and ATP-binding cassettes (ABCs) that drive the transport of various metabolites, lipids/sterols, and drugs [32]. Although these genomic sequences provide valuable data, the genome analyses have revealed contradictory data and inconsistencies between the annotated gene models and transcriptome evidence [31, 36]. Notoriously, those findings are related to sequencing platforms, which have been applied to having different strengths and weaknesses and the use of different strategies and stringencies in gene prediction.

Notwithstanding its novelty, the major challenges for the generation of whole genomes of *Cryptosporidium* are the quality and the yielding of limited DNA. Indeed, this is a critical step, as it is hard to recover enough quantity of DNA (2.5×10^{-5} highly purified oocysts correspondent approximately 10 μg) from faeces from natural infections. A theoretical estimate of the DNA content of one oocyst is of 40 fg [187]; therefore, it is tricky and arduous to recover enough quantity of DNA (2.5×10^{-5} highly purified oocysts correspondent approximately 10 μg) from unculturable samples with the quality necessary for high-throughput sequencing. Non-cultured samples may introduce a level of uncertainty and possess limited metadata. The lower the quality of the initial genome sequence, the higher the likelihood of yielding a missing or misassembled genome. A recent study evaluated an alternative method of preparing faecal samples using the combination of salt flotation, immunomagnetic separation (IMS), and surface sterilisation of oocysts prior to DNA extraction. The method has shown promise when used for the genome sequencing of samples of *C. parvum* and *C. hominis* [36]. This challenging issue can be resolved using a novel approach of *Cryptosporidium* cell-free culture and new long-read sequencing techniques, which will likely be beneficial for improving data. Increases in the quality of the target DNA boost the depth of coverage of the genome in higher levels, so base calls can be made with a higher degree of confidence. Also, the ability to culture relevant *Cryptosporidium* isolates in vitro, the development of novel gene-editing tools (knockout genes, CRISPR/Cas9, and RNAi), and 'omic' research (genomics, transcriptomics, and proteomics) represent essential paths towards significant advancements in the control of cryptosporidiosis.

6. Conclusions and future perspectives

Cryptosporidium is a major cause of diarrhoeal disease in humans worldwide, yet an effective therapy to eradicate the parasite is not available. Also, the diagnosis options remain limited in developing countries, which harm the surveillance and understanding of the epidemiology in resource-poor settings. In developed countries, large waterborne outbreaks in drinking and recreational water continue to occur, emphasising the need for better regulation and for improvements of drinking water treatment processes and control guidelines. However, in recent years, significant improvements have been achieved in understanding the key concepts

of the organism, mainly by increasing the use of molecular methods and genome sequences. Recent advancements in knowledge of *Cryptosporidium* provide the basis for the development of effective and practical strategies for the future prevention and control of cryptosporidiosis. The data from *Cryptosporidium* genome sequences have already improved our understanding of the metabolism and cellular processes. In fact, mining the genome and proteome data of *Cryptosporidium* will allow the development of new classes of compounds and molecular targets. However, it is worth underscoring the need for community-wide efforts to generate and integrate high-quality functional datasets that span the full spectrum of biology and life cycles in order to improve the predictive nature of models generated from large-scale system-based resources. Transcriptomes and proteomics from different growth stages are starting to be generated and promise to provide further insight into the biology of *Cryptosporidium*. Also, future studies will require careful validation and follow-up of each finding using in vitro and animal model studies.

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Conflict of interest

The authors declare that there is no conflict of interest.

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