Cryptosporidium: Host-Parasite Interactions and Pathogenesis

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Abstract

Purpose of Review *Cryptosporidium* spp. (*C. hominis* and *C. parvum*) are a major cause of diarrhea-associated morbidity and mortality in young children globally. While *C. hominis* only infects humans, *C. parvum* is a zoonotic parasite that can be transmitted from infected animals to humans. There are no treatment or control measures to fully treat cryptosporidiosis or prevent the infection in humans and animals. Our knowledge on the molecular mechanisms of *Cryptosporidium*-host interactions and the underlying factors that govern infectivity and disease pathogenesis is very limited.
Recent Findings Recent development of genetics and new animal models of infection, along with progress in cell culture platforms to complete the parasite lifecycle in vitro, is greatly advancing the *Cryptosporidium* field.
Summary In this review, we will discuss our current knowledge of host-parasite interactions and how genetic manipulation of *Cryptosporidium* and promising infection models are opening the doors towards an improved understanding of parasite biology and disease pathogenesis.

Keywords *Cryptosporidium* · Genetics · Infection models · Parasite biology · Host-parasite interactions · Pathogenesis

Introduction

The protozoan parasite *Cryptosporidium* spp. (*C. hominis* and *C. parvum*) is recognized as a leading cause of diarrhea and mortality in young children and immunocompromised individuals globally [1–3]. Repeated episodes of cryptosporidiosis in children living in resource-poor settings have been associated with malnutrition and growth defects [1, 4, 5]. While *C. hominis* infects only humans, *C. parvum* is a zoonotic pathogen and can infect both humans and animals [6]. *Cryptosporidium parvum* is an important veterinary parasite and a major cause of diarrheal disease in ruminant livestock, especially neonatal calves [7–9]. Transmission of *Cryptosporidium* infection occurs via the fecal-oral route upon ingestion of oocysts from contaminated food or water or via animal contact. These thick-walled infectious oocysts are resistant to routine disinfection procedures such as chlorination, thus making

them difficult to eliminate from swimming pools, animal housing facilities, and the environment [8, 10]. Due to their resilience to disinfection and highly infectious nature of oocysts, large outbreaks have occurred as a result of contamination of drinking water supply, and frequent outbreaks associated with treated recreational water facilities are reported from developed countries [11–13].

There are no drugs to effectively treat cryptosporidiosis and no vaccine to prevent the infection in young children, HIV/AIDS patients, and animals [14–16]. The only available and FDA approved drug, nitazoxanide, has limited efficacy in children and is not effective in immunocompromised individuals [17, 18]. Thus, it is critical to gain an in-depth understanding of *Cryptosporidium* biology and host-parasite interactions in order to develop effective drugs and vaccines to curb cryptosporidiosis. There have been several recent major technological advances in the field of *Cryptosporidium* that is rapidly expanding our fundamental knowledge on parasite lifecycle and disease pathogenesis. These include the development of molecular genetics to manipulate the parasite genome using clustered regularly interspaced short palindromic repeat/CRISPR associated protein 9 (CRISPR/Cas9) system that has unveiled new aspects of parasite biology and validation of drug targets, promising in vitro models for parasite propagation and new animal infection models to study host-parasite interactions [19••, 20••, 21••, 22••, 23••, 24•, 25, 26, 27•].

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The simple life cycle of Cryptosporidium

The lifecycle of *Cryptosporidium* is simple since both the asexual and sexual stages are completed within a single host, and the target of infection is the intestinal epithelial cell in the case of *C. parvum* [28, 29]. This is in contrast to related apicomplexan parasites such as *Toxoplasma gondii* and *Plasmodium falciparum* that have a complex life cycle which requires separate hosts to complete asexual and sexual development, and also these parasites are capable of infecting multiple cell types.

Interestingly, *Cryptosporidium* has a unique blend of features that it has adapted from *Plasmodium* and *Toxoplasma* as well as from gut-infecting gregarine apicomplexan parasites of invertebrates during evolution [30–33]. Although there are differences in terms of host cell specificity and lifecycle completion, *Cryptosporidium* has conserved features that are typical of apicomplexans such as apical secretory organelles (rhoptry, micronemes, and dense granules) for parasite invasion, as well as similar replicating and cyst stages. However, it lacks many components of the moving junction machinery that allows active invasion of *Toxoplasma* and *Plasmodium* and has also lost its apicoplast and mitochondrion and is dependent on glycolysis for its energy requirements [34, 35]. The absence of some conserved invasion components is not surprising, since *Cryptosporidium* does not become totally intracellular after invasion, but displays this peculiar epicellular localization upon encapsulation by the host cell membrane [31, 32]. The molecular mechanisms from both the host and parasite side that lead to this epicellular niche are not known.

The *Cryptosporidium* lifecycle begins with the oral ingestion of infective form, the thick-walled oocysts, and each oocyst contains four sporozoites [36]. These oocysts undergo excystation due to change in temperature, pH, action of bile salts, and parasite proteases, along with other unknown triggering factors. The released sporozoites glide and invade intestinal epithelial cells (enterocytes) and transform into a uninucleated trophozoite. Trophozoites undergo three rounds of asexual replication (merogony) to produce a mature type I meront with eight merozoites. Merozoites released from the type I meront invade adjacent intestinal epithelial cells to yield additional type I meronts or transform into type II meronts that contain four merozoites. Merozoites from type II meronts are thought to transform into the sexual stages, the macrogamont (female) and microgamont (male). The macrogamont has a large, eccentric nucleus and in its cytoplasm stores amylopectin granules, lipid vacuoles, and wall forming bodies. The microgamont gives rise to 16 non-flagellated, bullet-shaped male gametes that find female gametes by unknown mechanisms, resulting in fertilization [36, 37]. The fusion of the male and female nuclei results in the formation of a diploid zygote that undergoes meiosis and sporogony to form four haploid sporozoites within an oocyst. This sporulated oocyst is then passed in the feces to be taken up by another host for the cycle to continue.

Each sequential step in the *Cryptosporidium* developmental cycle has to be precisely programmed for the successful completion of its lifecycle. This highly regulated programming has been demonstrated by time course infection experiments and immunofluorescence microscopy in cell culture by infecting human ileocecal adenocarcinoma cells (HCT-8) with genetically engineered *C. parvum* strains and also by using a combination of 5-ethynyl-2′-deoxyuridine (EdU) labeling and monoclonal antibodies against different parasite stages [37–40]. Asexual stages have been reported to be predominant up to 36 h of infection, and after that, there is a dramatic shift towards development of sexual stages. At 36 h post infection, microgamonts begin to emerge, followed by macrogamonts and these stages are abundant at 48 h of infection [37, 39]. Although we now recognize that parasite development is timed and all steps occur in a coordinated manner, we still do not know the signals that the parasite senses and the regulatory mechanisms that control progression from one stage to another. Transcriptomics studies have revealed a stage-specific expression of *Cryptosporidium* genes during the parasite developmental stages. There are clearly different gene expression profiles at asexual stage versus female gametocytes; and genes required for genetic recombination, amylopectin, trehalose metabolism and oocyst wall formation have been reported to be highly upregulated in the female gametocytes [37, 41]. Recent studies have also reported the emerging role of long non-coding RNAs (lncRNA) delivered by *Cryptosporidium* into the host cell to manipulate host gene expression and pathogenesis [42–45].

The requirement of *Cryptosporidium* to complete the entire lifecycle in a single host has been challenging to mimic in the laboratory for continuous culture of the parasite. The commonly used HCT-8 cells only allow a short window of 72 h to maintain *C. parvum* growth, and the culture arrests at this point with no new oocyst formation. Using the power of CRISPR/Cas9 genome editing, reporter strains were generated to track *Cryptosporidium* development in HCT-8 cells and in IFN-gamma knockout (IFN-γ KO) mice [37]. After 48 h of infection, sexual stages of the parasite were reported to predominate in culture, but fertilization of male and female gametes did not occur. This block in fertilization could be overcome in infected IFN-γ KO mice, where productive fertilization of gametes resulted in oocyst formation [37]. There have been increasing efforts in recent years for the development of in vitro systems that can allow completion of the entire lifecycle of the parasite in the laboratory and sustain long-term growth for viable oocyst production [24•]. These include three-dimensional bioengineered system utilizing immortalized cancerous Caco-2 and HT29-MTX cells, human intestinal enteroids (HIE) derived from intestinal tissues, human

small intestine and lung organoids, and stem-cell derived cultures of mouse intestinal epithelial cells under air-liquid interface (ALI) conditions [21••, 23••, 46, 47]. In the ALI culture system, transgenic fluorescent parasite lines were generated using CRISPR/Cas9 editing and used to demonstrate genetic crossing *in vitro* [21••]. Furthermore, the long-term growth of *C. parvum* in ALI culture system has been employed to test anti-cryptosporidial compounds for time-dependent killing of the parasite in order to define cidal versus static activity of these compounds [39].

Cryptosporidium—host cell interactions

The interaction of *Cryptosporidium* with its host is complex; and an interplay of several parasite and host factors determines disease pathogenesis or protection from infection (Fig. 1). Therefore, it is critical to understand these interactions in order to identify key biological mechanisms that can be targeted for the future development of effective vaccines and drugs against cryptosporidiosis. However, our current knowledge on parasite attachment, invasion, replication, and gametocyte development is very limited.

On the parasite side, there are only a handful of factors that have been identified until date to play a role in host-parasite interactions [29, 48]. These include the thrombospondin related adhesive protein (TRAP-C1), circumsporozoite-like protein (CSL), P23, CP47, Cpa135, CPS-500, mucins (CpMuc4, CpMuc5) and mucin-like glycoproteins GP900, GP60 (proteolytically cleaved into GP40/15 mature glycopeptides), and C-type lectin (CpClec) [29, 48–57]. These proteins have been reported to localize to the apical end or on the surface of sporozoite, and many of these are shed in trails during gliding motility of sporozoites. Thus, based on their localization, binding to host cell or antibody-based inhibition of infection, these proteins have been implicated to play a role in the initial attachment and invasion process [48, 51, 58]. We still do not fully understand the gamut of parasite secreted proteins that interact with the host for a productive zoite attachment and invasion process, and the molecular mechanism underlying these interactions. Moreover, it is not known if the

genes encoding for these *Cryptosporidium* proteins during the invasion process, proliferating asexual stages, and gametogenesis are essential for parasite survival. These challenges can now be overcome due to the availability of molecular tools to genetically manipulate the parasite genome and conditional protein degradation system that allows investigation of essential gene function [27•, 59].

On the host front, a key role of signaling and cytoskeletal remodeling resulting in accumulation of host filamentous actin upon *Cryptosporidium* invasion at the interface of this interaction has been reported [60–63]. Another cellular structure that has been identified is the membranous "feeder organelle" at this interface, and this organelle is thought to function in the uptake of host metabolites by the parasite. The parasite has reduced biochemical synthesis pathways and lacks enzymes for synthesis of amino acid, sugars, and nucleotides, but has many transporters encoded in its genome [34, 35, 64]. Although it has a highly streamlined metabolism, *Cryptosporidium* can directly acquire metabolites from the host or the gut environment. A recent study has identified neonatal mouse gut metabolites and their role in modulating *C. parvum* growth in vitro [65]. Medium or long-chain saturated fatty acids were reported to inhibit parasite growth, while omega-3 and omega-6 polyunsaturated fatty acids promoted parasite invasion and growth [65].

The parasite can also scavenge some precursor metabolites and encodes enzymes to convert these precursors and generate nucleotides, fatty acids, and amylopectin. Many of these conversion enzymes in *Cryptosporidium* such as the thymidine kinase (TK) and inosine monophosphate dehydrogenase (IMPDH) for nucleotide pathways and type I fatty acid synthesis enzymes have been acquired from bacteria via horizontal gene transfer making them attractive drug targets [66, 67]. CRISPR/Cas9-mediated genetic manipulation of *Cryptosporidium* has allowed targeted deletion of genes encoding for TK and dihydrofolate reductase-thymidylate synthase (DHFR-TS) and revealed the role of TK in providing an alternative route to pyrimidine nucleotide synthesis in the absence of DHFR-TS [19••, 26, 68]. Also, multiple enzymes in the single purine nucleotide synthesis pathway such as IMPDH, GMP synthase (GMPS), adenosine kinase (AK), and the adenosine transporter (AT) can be genetically ablated without any effect on parasite growth, thus demonstrating that the parasite imports purine nucleotides from the host cell [26].
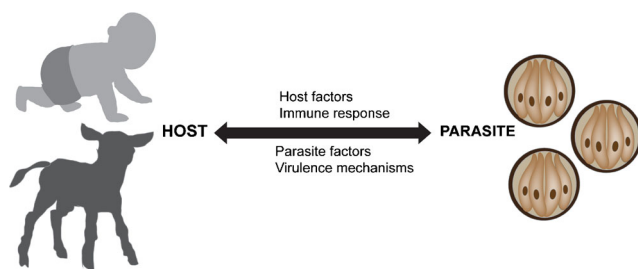
Animal models of infection to study virulence, host-parasite interactions, and disease pathogenesis

Neonatal calves can be naturally infected with *C. parvum* and thus serve as an ideal model to assess clinical illness signs of cryptosporidiosis. The calf model has been successfully used

Fig. 1 Interactions between *Cryptosporidium* and host determines pathogenesis or protection from disease. *Cryptosporidium parvum* oocysts (parasite) and hosts are shown

for evaluating the protection potential of colostrum, recombinant parasite proteins, and monoclonal antibodies in passive immunization studies as well as for testing the therapeutic efficacy of anti-cryptosporidial compounds [8, 69–72]. For *C. hominis*, the gnotobiotic piglet model of acute diarrhea has been the only available model for human cryptosporidiosis due to the high similarity of anatomy, physiology, and immunology between pigs and humans and clinical diarrheal signs [73].

Although these are efficient infection models for studying vaccine potential, they come with their own challenges such as requirement of specialized handling facilities for infection and challenge studies. On the flip side, small animal models such as immunocompromised IFN-γ KO mice that are relatively easy to handle are not suited to understand immune responses to *Cryptosporidium* infection and the key players that provide protection from disease. Thus, the lack of facile immunocompetent rodent models has been a major roadblock for evaluating the potential of putative virulence antigens in infectivity, disease pathogenesis, and immune protection. Moreover, there has been no single animal model that can be used to test for both *C. parvum* and *C. hominis* immunogens.

These two roadblocks have been overcome recently by breakthrough studies that report development of two animal models of infection, utilizing immunocompetent mice and rats to mimic human disease progression. The immunocompetent mouse model is based on using a naturally isolated and laboratory-adapted *C. tyzzeri* strain that can be genetically manipulated to track disease progression and study immune correlates of protection [20••]. *Cryptosporidium tyzzeri* strain infects the small intestine of C57BL/6 immunocompetent mice and closely resembles *C. parvum* and *C. hominis* in terms of its high sequence identity at the nucleotide level and similar intestinal pathology such as villus blunting, crypt hyperplasia, and lymphocyte aggregation as observed with human cryptosporidiosis.

Using CRISPR/Cas9 genome editing, the *C. tyzzeri* strain was genetically engineered to create a reporter parasite strain that enabled measurement of parasite burden in mouse over time. This natural mouse model in which both host and parasite are genetically tractable has allowed to unravel the role of IFN-γ during early stage of infection and the pivotal role of T cells in parasite clearance [20••, 74]. Genetically engineered *C. tyzzeri* strains have also been instrumental in defining the innate mechanism for control of *Cryptosporidium* infection by NOD-like receptor family pyrin domain containing 6 (NLRP6) inflammasome dependent release of the pro-inflammatory cytokine IL-18 [75]. The *C. tyzzeri* mouse model will allow future studies to assess the immunogenic potential of vaccine candidate antigens by uncovering the key molecular interactions between the parasite and the host, immune responses to *Cryptosporidium*, and the immunoregulatory mechanisms that confer development of protective immunity.

Recently, an immunocompetent rat model for *C. parvum* and *C. hominis* has been developed for testing of future vaccine candidate antigens [22••]. This model used the intratracheal route to deliver *C. parvum* and *C. hominis* sporozoites to successfully infect tracheal epithelial cells and reported the generation of a *Cryptosporidium*-specific immune response. The systemic antigen-specific response was assessed by IFN-γ production, while the humoral immune response was evaluated by serum IgM and IgG production at day 10 and day 14 post infection. The applicability of this model for vaccine studies was evaluated by performing challenge studies. Rats infected with *C. parvum* sporozoites that had cleared infection showed complete protection against disease upon re-infection by intratracheal inoculation, thus demonstrating the suitability of this model for vaccine development [22••].

Conclusion

Advances in genetics, cell culture platforms, and new animal infection models are providing valuable insights into the basic biology of *Cryptosporidium*. These technological advancements will allow us to understand the molecular underpinnings of host-parasite interactions, mechanisms of generation of immune response against *Cryptosporidium* and disease pathogenesis, for the future development of a vaccine against cryptosporidiosis.

Declarations

Conflict of Interest Derek Pinto and Sumiti Vinayak declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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•• Of major importance

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