**Stem cell-derived enteroid cultures as a tool for dissecting host-parasite interactions in the small intestinal epithelium**

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**Abstract**

*Toxoplasma gondii* and *Cryptosporidium* spp.can cause devastating pathological effects in humans and livestock, and in particular to young or immunocompromised individuals. The current treatment plans for these enteric parasites are limited due to long drug courses, severe side effects, or simply a lack of efficacy. The study of the early interactions between the parasites and the site of infection in the small intestinal epithelium has been thwarted by the lack of accessible, physiologically relevant, and species-specific models. Increasingly, 3D stem cell-derived enteroid models are being refined and developed into sophisticated models of infectious disease. In this review we shall illustrate the use of enteroids to spearhead research into enteric parasitic infections, bridging the gap between cell line cultures and in *vivo* experiments.

**Key words**: Enteroid; Organoid; Intestinal epithelium; Apicomplexan; *Cryptosporidium parvum; Toxoplasma gondii*; *Neospora caninum*

**Introduction**

The Apicomplexa are a phylum of intracellular protozoan parasites are among the most prevalent morbidity-causing pathogens worldwide. Within this phylum, lie a number of intestinal protozoa of medical and veterinary importance, including *Toxoplasma gondii*, *Neospora caninum* and *Cryptosporidium spp*. All of these parasites can be acquired orally, before infecting or invading the intestinal epithelium. *Toxoplasma gondii*, and *Neospora caninum* will disseminate, causing non-specific systemic symptoms during acute infection, before encysting in muscles and the nervous system. *Cryptosporidium spp.* remain within the epithelium, causing gastrointestinal symptoms. Historically, a lack of suitable experimental models has limited our understanding of very early interactions between these parasites, and the multiple cell lineages of the small intestinal epithelium. Here, we review recent progress made in 3D stem cell-derived enteroid models in characterising the interactions that occur between these parasites and the host intestinal epithelium.

Enteroids are 3D tissue culture models derived from Lgr5+ stem cells that reside at the base of the crypts of the small intestinal epithelium. When cultured in Matrigel, with a cocktail of growth factors, Lgr5+ stem cells will proliferate and differentiate to form 3D structures with bud-like crypts and villus domains, enclosing a central lumen (Figure 1A-B)1. Crucially, these cultures contain a representative mixture of the differentiated cell types that make up the intestinal epithelium, including goblet cells, enteroendocrine cells, Paneth cells, tuft cells and enterocytes. Because of this, enteroids are increasingly being used to study or propagate enteric pathogens, including norovirus, rotavirus, *Salmonella*, and *Escherichia coli*2-8. In fact, enteroid models have now been developed for multiple different species, meaning that a variety of infectious agents can be studied in their natural hosts (Figure 1B-D)9.

One technical issue is that while enteric infections occur at the luminal surface of the small intestinal epithelium, enteroids feature an enclosed lumen, making the large-scale application of pathogens a challenge. The small intestinal epithelium is polarised, with different receptors at both the basal and apical sides1. Therefore, infection from the apical surface may incur different host responses to that of the basal side. Development of enteroid-derived monolayers or “inside-out” enteroid models will be important in addressing this issue (Figure 1E-F)10-15.

Enteroid models have recently been applied to the study of *Toxoplasma gondii* and *Cryptosporidium spp*, yielding important biological insights14,16-25. Since enteroids recreate many of the characteristics of the *in vivo* environment in a sustainable and cost-effective way, they pave the way for a reduction in the use of animals in research into these important parasites.

***Toxoplasma gondii***

*Toxoplasma gondii* is an obligate intracellular parasite, prevalent in most warm-blooded animals26. *T. gondii* infections in humans tend to be asymptomatic or associated with mild flu-like illness, however as an opportunistic parasite it is capable of causing devastating effects, frequently manifesting in the development of brain abscesses and encephalitis27,28. There is a vast socio-economic impact of toxoplasmosis, especially due to the cost of care for those with mental impairment and blindness as a result of congenital infection29. *T. gondii* is also responsible for economic losses in the agricultural sector due to its pathogenic impact in livestock. Particularly in sheep, the parasite has been shown to lead to reproductive failures which includes abortion, stillbirth and neonatal mortality30,31.

*T. gondii* is tissue cyst-forming and uses a prey-predator system in which it alternates between intermediate and definitive hosts for asexual and sexual reproduction, respectively. Not only can *T. gondii* be transmitted between intermediate and definitive hosts but also between intermediate hosts via carnivorism or even between definitive hosts32. *Felidae* family members are the definitive hosts of *T. gondii* and sexual reproduction of the parasite occurs in the small intestine of the cat33. Bradyzoites invade the epithelial cells of the small intestine where they initiate the formation of numerous asexual generations before the sexual cycle begins. Sexual development then follows with the formation of both male and female gametes, which fuse and fertilise each other34. Oocysts are then formed after fertilisation, within enterocytes, which are subsequently liberated from the cell and excreted as the unsporulated form in cat faeces35. Following sporulation in the environment, sporozoites become infective. Oocysts can be ingested from contaminated pasture, produce or water by intermediate hosts, and following excystation, sporozoites rapidly infect the small intestinal epithelium, entering the underlying lamina propria within 2 hours33,36. Sporozoites differentiate into the rapidly dividing tachyzoite form during the first few hours of infection. Infection may also be initiated following consumption of tissue cyst-containing meat from other intermediate hosts. In this case, bradyzoites are released from cysts during transit through the gastro-intestinal tract, and can be observed within epithelial cells by 1 hour post infection37.

Although the vast majority of non-congenital infections occur through the oral route, our understanding of how the parasite interacts with the small intestinal epithelium, and how this interaction influences protective immunity, remains limited. This is largely due to a historical paucity of appropriate and amenable model systems. Recently developed enteroid co-culture models can help to bridge this gap.

**Selection of appropriate model systems for studying *T. gondii* infection of the small intestinal epithelium**

There are several important considerations when selecting an appropriate model system for studying *T. gondii* infection of the small intestinal epithelium. Firstly, the dynamics of *T. gondii* infections depend significantly on the initiating lifecycle stage. To best mimic a natural infection during *in vivo* studies, oral infections using tissue cysts or oocysts are ideal. Tissue cysts (derived from the brains of chronically infected mice) are more commonly used due to the difficulty and expense of sourcing oocysts from infected felines, and increased biosafety concerns as the oocysts can withstand many decontamination procedures. A drawback of oral infection models is that parasites only become readily detectable in the small intestine around 5 days post-infection38,39. Before this, it is challenging to investigate host epithelial responses to rare foci of infection, against a background of largely unperturbed tissue. Tachyzoites, grown readily in tissue culture, have also been used to initiate oral infection40. Although they need to be given in high doses to avoid destruction in the stomach, and do not represent a natural oral infection, they do generate relatively high proportions of infected epithelial cells soon after infection40,41. Tachyzoites tend however to be given via the intra-peritoneal route. While this obviously bypasses the intestinal epithelium and may lead to altered infection outcomes, it should be acknowledged that this model has been instrumental in characterisation of many aspects of the host immune response to *T. gondii*41,42*.* The use of enteroid models addresses many of these issues, by allowing for investigation of the earliest epithelial responses, to biologically relevant parasite life stages.

Secondly, the species and genetic background of the host animal has an impact on infection dynamics. The most widely used model to study oral infections is the C57BL/6 mouse model. Unlike the BALB/c mice, which develop chronic infection, C57BL/6 are highly susceptible to *T. gondii,* often succumbing to Th1-mediated intestinal inflammation during the acute phase of the disease43. While intestinal inflammation has been documented in other species it is not a common feature of acute infection in humans or farm animals, and caution is warranted when studying epithelial responses in these mice44. The ability to generate enteroid cultures easily from multiple species and from mice of multiple genetic backgrounds will be beneficial in this respect14,17,18,45.

A third issue, affecting mostly *in vitro* models, is how well the cell culture represents the complexity of the intestinal epithelium *in vivo*. Knowledge of *T. gondii* interactions with the small intestinal epithelium derive from *in vitro* cultures with cell lines such as Caco-2, which provides a monolayer of polarised enterocyte-like cells and is routinely used for investigations of intestinal physiology. Caco-2 cells have limited cellular complexity when compared to enteroids or the intestinal epithelium *in vivo*, differentiating towards absorptive enterocyte-like cells, and lacking secretory lineages such as goblet cells. In addition, Caco-2 cells originally derive from the human colon, while *T. gondii* targets the small intestinal epithelium46,47. Immortalised epithelial cell line cultures can be more generally problematic: accumulation of genotypic and phenotypic changes over serial passage means that they fail to properly recapitulate the physiology of the normal intestinal epithelium48 49. A particularly relevant example for infection studies are reports of altered cytokine receptor expression and responsiveness in intestinal epithelial cell lines compared to freshly isolated epithelium50. As a result, most of the research performed using these types of cell lines must be confirmed in primary cells. Explant culture of intestinal tissue *in vitro* allows for increased cellular diversity and an intact 3D tissue architecture. However, these cultures are only viable for a short period of time due to inadequate oxygenation of the interior of the tissue. Therefore, this model is limited to short term infection studies while also relying on repeated animal biopsies51-53.

Stem cell-derived enteroid cultures are a particularly useful alternative, and address many of these shortcomings. They contain a representative mixture of the differentiated cell types that make up the intestinal epithelium, together with a 3D crypt-villus domain organisation, and can be maintained in culture for long periods of time54,55. Undoubtedly, the enteroid model still has some limitations in application. This is chiefly due to the absence of other tissue features, such as a vasculature, microbiota, and an immune cell compartment, that influence epithelial function. The heterogeneity of the enteroid population in terms of size and differentiation may also fundamentally influence experimental results, while may also have an impact of the results observed across experiments56. Finally, the cost of specialist growth media for maintaining enteroid cultures may be prohibitive for some research groups57.

**Multiple routes of invasion of the intestinal epithelium by *T. gondii***

Apicomplexan mechanisms of target cell invasion are distinct from other intracellular microorganisms. Active gliding motility allows the parasite to attach to the host cell with its apical end. Unlike most viruses and intracellular bacteria, *T. gondii* then actively penetrates the host cell using actin-based motility, and forms a parasitophorous vacuole58. The exact mechanism used by *T. gondii* to cross the intestinal epithelium *in vivo* remains unclear, but a number of potential mechanisms have been explored in epithelial cell line cultures.

The first method *T. gondii* may use to invade the intestinal epithelium is transcellular traversal, where the parasite actively penetrates the apical cell membrane and exits through the basal side59,60. This process is analogous to that described in basic cell culture models (e.g. human foreskin fibroblasts) and involves the secretion of proteins from secretory organelles: the rhoptries, the micronemes and the dense granules at the apical tip of the parasite61. This is consistent with early ultrastructural studies showing sporozoites and bradyzoites within parasitophorous vacuoles inside epithelial cells, early after oral infection in mice36,37.

The second method potentially used by *T. gondii* to cross the intestinal epithelium is the paracellular route. In *in vitro* studies using epithelial cell lines, or explanted tissue, parasites were shown to cluster near cellular junctions, prior to migrating between epithelial cells47,62. The parasite appears to achieve this without altering barrier integrity, though it does redistribute occludin from the tight junction, while also changing the abundance of a number of other tight junction-associated proteins47,63-65. It is believed that this mode of migration is aided by interactions between parasite MIC2, and host ICAM-1, which is upregulated during *T. gondii* infection59.

The third, less well described, method is the Trojan horse mechanism, which has been shown using murine oral infection models. Oral infections with *T. gondii* result in the transepithelial migration of neutrophils, and the parasite may then exploit the neutrophils to transfer across the epithelium38.

The exact method used may be linked to parasite strain: type 1 strains have long distance migratory phenotype, and more readily transmigrate across epithelial monolayers via the paracellular route62. By contrast, type 2 strains are more proficient at inducing hyper migratory phenotypes in immune cells, suggesting they could favour a trojan horse mechanism66. While it would be technically challenging to directly visualise the invasion of parasites across the intestinal epitheliumin real time using an animal model, enteroids would allow comparative studies of infection routes used by different parasite strains.

**Defensive function of the intestinal epithelium against *T. gondii* infection**

The epithelial cells which line the intestine provide a physical barrier protecting the host from the luminal contents. It has been demonstrated that *T. gondii* initiates activation of the NLRP3 inflammasome in epithelial cells which leads to the secretion of IL-1β in a purinergic P2X7 receptor-dependent manner (P2X7R). The production of IL-1β by intestinal epithelial cells precipitates a pro-inflammatory response during infection which leads to control of *T. gondii* proliferation67.

Goblet cells secret mucins to form a protective layer on the intestinal epithelium. During a *T. gondii* infection there is increased production of mucins, resulting from increases in both Alcian blue+ goblet cells (which secrete more acidic mucins) and Periodic Acid Schiff+ goblet cells (neutral mucins). This shift to acidic mucins is thought to promote the expulsion of *T. gondii,* while the increase of cell numbers is an attempt to thicken the protective mucus layer68.

Paneth cells can release antimicrobial proteins and peptides, including defensins and lysozyme, into the lumen of the intestine. *T. gondii* infection results in the up -regulation of TLR9 expression in epithelial cells. Stimulation via TLR9, either by unmethylated CpG motifs in the parasite itself, or via the intestinal microbiota, results in production of type 1 IFNs, which drive α-defensin production and degranulation in Paneth cells69,70. However, certain strains of *T. gondii* have been shown to downregulate the production of defensins, although the precise mechanisms and effector molecules involved remain unclear71. Although defensins have a limited impact on *T. gondii* directly, they effect the early control of the infection through the promotion of the protective Th1 immune response69,72-74 .

**Stem cell-derived enteroids in the study of *T. gondii* infection**

Stem cell-derived enteroid models bridge the gap between *in vitro* cell line cultures and *in vivo* models to provide researchers with a more reliable, ethical, and species-specific model to study enteric infections. This technology not only allows for the verification of previously discovered *T. gondii* molecular mechanisms, but it can also yield novel biological insight, and act as a platform for drug development (Table 1).

To reflect the physiologic route of infection via the apical epithelial surface, we have developed three enteroid-based techniques for modelling *T. gondii* infection: fragmentation, micro-injection and open-format enteroid cultures14. Fragmentation of enteroids increases the probability of *T. gondii* infecting through the newly exposed apical epithelial surface. However, although this method exposes the apical surface it does not limit infection to that site14,16,75. One way to restrict infection to the luminal surface is through the use of a microinjection system that delivers pathogens directly into the lumen of organoids4*.* However, while successful for bacteria and viruses, this approach is technically challenging for larger organisms, like *T. gondii*14. An alternative method of exposing the luminal surface for infection is the collagen-supported epithelial sheet model, where 3D enteroids are passaged onto collagen gels14,76. Instead of reforming 3D structures, the epithelial cells grow as a monolayer on the surface of the gel. Importantly, the epithelium is polarised, and retains a range of differentiated epithelial cell types, with crypt-villus domain organization14. The ability of this model to support *T. gondii* infections has been established using multiple *T. gondii* strains including the virulent RH and avirulent VEG strains. The collagen-supported epithelial sheets also give a real time insight into invasion and replication of *T. gondii.* Parasites were initially observed in parasitophorous vacuoles as early as 1-hour post infection, while parasite replication was first detected between 16-24 hours post infection14. This model allows for proteomic analysis of the host epithelial response, as well as assessment of the effect of drugs or other perturbagens on parasite invasion and replication14.

Felines are the definitive host of *T. gondii,* and the sexual stage of the parasite lifecycle is restricted to the feline small intestinal epithelium. Only recently have researchers defined the long sought after mechanism of species specificity, with cat enteroids playing an integral role in the study18. Felines are the only mammals to lack delta-6-desaturase activity in their intestines, resulting in an excess of linoleic acid. Linoleic acid enhances progression through the sexual stages. It has been determined that the sexual stages of the *T. gondii* lifecycle can be initiated in feline enteroid cultures supplemented with linoleic acid. Remarkably, the sexual cycle of *T. gondii* was demonstrated in mice through supplementing linoleic acid in the diet while inhibiting delta-6-desaturase18. This ground-breaking study broke the species barrier of *T. gondii* sexual reproduction.

*T. gondii*’s fast replicating tachyzoite stage is widely used in *in vitro* infection models. Further development of multi-species enteroid models, featuring bradyzoites and sporozoites as initiating life stages, will be crucial moving forward. In addition, intestinal dendritic cells (DC) play an important role both in protective immunity to *T. gondii* infection, and in dissemination of the parasite. Further development of co-culture models of enteroids with DCs will allow us to determine how epithelial crosstalk influences DC function, and how the parasite alters these interactions (Figure 2).

***Neospora caninum***

*Neospora caninum* was mistakenly identified as *Toxoplasma gondii* when it was first observed in dogs in Norway in 198477. These protozoan parasites have similarities in morphology, and both cause abortion, reproductive failure, and potentially neonatal mortality in livestock78. However, unlike *T. gondii*, *N. caninum* uses canines as its definitive host, and is most commonly associated with abortion and reproductive failure in cattle. A further striking difference between the two is that there is no evidence to date that *N. caninum* is capable of infecting humans79. There is currently no licenced or effective veterinary vaccine for *N. caninum.* Treatment programs attempt to control the clinical manifestations of the infection instead of curing the animal of the parasite80. Although the mechanisms of infection are very similar to *T. gondii*, unfortunately less is known about the exact interactions between *N. caninum* and the intestinal epithelium. However, future research using species-specific enteroid models should allow us to bridge these gaps of knowledge. In particular, recently developed bovine enteroid models have been shown to be susceptible to infection with *N. caninum* NC-LIV strain (our own unpublished data)14,45,81.

***Cryptosporidium* *spp.***

*Cryptosporidium* is a monoxenous, coccidian parasite that invades the epithelial cells of the small intestine. *C. parvum* is the most common pathogenic species causing cryptosporidiosis in both animals and people. The parasite inhabits a parasitophorous vacuole: an intracellular but extra-cytoplasmic structure that provides the parasite protection from the cell's defensive responses82. Its distinctive feeder organelle, only present during the intracellular stages, is believed to be an interface between the parasite and the host cell, allowing uptake of nutrients without interacting with the hostile environment of the gut83. *C. parvum* causes severe and even fatal diarrheal disease in immunocompromised adults, young children, and neonatal ruminants. With 40% of calf diarrheal disease attributed to *Cryptosporidium* alone and a further 20% caused by *Cryptosporidium* co-infections, this parasite presents a large-scale economic and welfare issue for the farming industry83. The lack of a vaccine and effective treatments calls for the development of new therapeutic regimes, rendering the study of interactions between *Cryptosporidium* and the native intestinal epithelium, imperative. Our understanding of how the small intestinal epithelium protects itself against infection is relatively poor due to a lack of relevant *in vitro* models that replicate the *in vivo* setting and allow completion of the lifecycle. With the recent development of enteroid models, new systems for the study of interactions between *Cryptosporidium*, the host epithelium, immune cells and commensal bacteria are now possible, bringing with them, new insights into how to combat this parasite in both humans and animals.

***In vitro* generation of infectious *C. parvum* oocysts**

The lifecycle of *Cryptosporidium* is complex and multi-staged with asexual and sexual phases and has been challenging to bring to fruition *in vitro* due to a lack of long-term culture models. It has been attempted in several systems including primary and cancer cell lines, but more recently has been successfully completed in enteroid models84.

Human ileocecal adenocarcinoma cells (HCT-8) have been a major cell line commonly used in *Cryptosporidium* research as the parasite is able to complete its lifecycle in these cells85. However, it does not produce sufficient numbers of infectious oocysts to sustain infected cultures. The most recent advance in cancer cell lines, COLO-680N cells, allow the production of infectious oocyst for up to 8 weeks, making it far superior in terms of longevity and quantity of infectious oocysts produced compared to the HCT-8 cell line82,86. Recently, both asexual and sexual phases of the lifecycle were successfully completed using several new methods (Table 2). One novel method which cultured Caco-2 and HT29-MTX cells on a 3D silk scaffold, was able to propagate all stages of *Cryptosporidium* for up to 15-17 days87. Another technology, using HCT-8 cells lining a hollow fibre bioreactor (HFB), is promising in the continuous production of *C. parvum* oocysts for up to 20 weeks. This system simulates *in vivo* conditions by supplying oxygen and nutrients to host intestinal cells from the basal surface and enables the formation of a low redox, high nutrient environment on the apical surface88. All of these cell lines are reviewed more extensively elsewhere82. While many of the cell lines used to propagate *Cryptosporidium* derive from intestinal epithelium, or other epithelial tissues, they lack the cellular diversity, architecture, and site specificity of the small intestinal epithelium *in vivo*, and are likely to be poor models of host defence to parasitic infection. This is where enteroid models truly break ground.

**The role of the microbiota in protection against *Cryptosporidium* infection**

*Cryptosporidium* infection is decidedly age-dependent, with the most severe disease, and greatest shedding of oocysts, observed in young children and in calves under 6 weeks of age. *Cryptosporidium* ordinarily penetrates no further than the intestinal epithelium, meaning that the epithelial response to infection is likely to be critical to determining the outcome. While significant development of the small intestinal epithelium takes place at birth, it continues to adapt to changes in nutrient and microbiota composition throughout life, and this adaptation may explain the decline in susceptibility to *Cryptosporidium* infection. Supporting this idea, scrapings of the intestinal mucosa of adult cattle, but not calves, can protect rats from *C. parvum* infection when inoculated orally89. Subsequent studies suggest the protective factor(s) are found within the cell membrane90. Interestingly, the intestinal mucosa of young calves that had previously been infected with *Cryptosporidium* did not transfer protection, again indicating that maturation of the microbiota and intestinal epithelium, rather than specific immunological memory, underlies resistance to infection. A mature, stable, microbial community can protect the host against invading pathogens by enforcing epithelial barrier function, for example by regulating expression of tight junction proteins, mucins, AMPs, and inflammatory mediators.

The role of the microbiome in resistance to *Cryptosporidium* has been investigated in a SCID mouse model, showing that SCID mice with a normal intestinal flora were significantly more resistant to *Cryptosporidium* infection than germ-free SCID mice91. In human volunteer infection studies, high pre-infection levels of faecal indole, associated with increased relative abundance of *E. coli*, *Bacillus spp.* and *Clostridium*, was predictive of resistance to *Cryptosporidium* challenge92. Indole acts on epithelial cells to increase barrier integrity and reduce expression of inflammatory mediators, though how it mediates resistance to *Cryptosporidium* remains unknown. In mouse models, murine isolates of *Lactobacillus reuteri*, increased resistance to *Cryptosporidium*93. Other studies are less convincing. Colonisation of mice with *Enterococcus faecalis* from silage led to only a modest reduction in *Cryptosporidium* infection in the ileum94. Lactic acid producing bacteria had no effect on resistance to infection in field trials in calves, while commercial probiotics had no effect in a suckling rat model95,96. Finally, a probiotic intended for human use actually enhanced susceptibility to *Cryptosporidium* in mice97. These conflicting studies demonstrate that different isolates of commensal bacteria can have opposing actions against *Cryptosporidium* infection, and that the specific effects of different bacterial isolates deserves further study. In this respect, recently described enteroid-anaerobe co-culture models, which maintain a physiologically relevant oxygen gradient across the epithelial layer, would prove particularly useful98.

**Defensive function of the intestinal epithelium against *Cryptosporidium* infection**

Once the parasite has penetrated the membrane of the epithelial cell, rapid defence mechanisms are launched by the epithelial cell that can work independently of the immune system. For example, epithelial cells may be triggered to secrete cytokines and antimicrobial peptides such as interferons (IFN), interleukins (IL) and β-defensins. Type I IFNs (IFN-α/β) are produced by epithelial cells in response to *Cryptosporidium* infection99,100. *Cryptosporidium* development was inhibited in Caco-2 and murine enterocyte CMT-93 cell lines, pre-treated with different subtypes of IFN-α/β. In the same study, BALB/c and SCID mouse models, treated with anti-IFN-α/β neutralising antibodies, had higher numbers of colonic oocysts than untreated mice showing that IFNα/β contributes to early innate immune responses99. However, in another study, there was increased expression of IFN-β (and IFN-λ) in IPEC-J2 monolayers but no significant increase in expression of IFN-α, suggesting the protective effects may be type specific101. These inconsistent results between studies could be explained by the use of different *in vitro* models, and could be verified in enteroid models21. Interestingly, there is an age-dependent increase in the ability of monogastrics and ruminants to produce type 1 IFN in response to viral infection, and this difference may also help to explain the increased susceptibility of young children and neonatal cattle to *Cryptosporidium* infection102-104.

The production of Type III IFN, or IFN-λ, by epithelial cells is often associated with viral infections such as rotavirus, however, has only recently been implicated in parasite infections. In the study previously mentioned, using an infected piglet model, *Cryptosporidium* infection was shown to cause the upregulation of IFN-λ3, and genes targeted by Type III IFN signalling. In the same study, suckling C57BL/6 mice were shown to conserve the IFN-λ3 response after infection with *C. parvum.* IFN-λ3 was therefore shown to promote epithelial defence and barrier function against *C. parvum* infection101.

The expression of TLR2 and TLR4 is upregulated during *C. parvum* infection leading to the increased activation of NFκB and, in turn, pro-inflammatory cytokines such as IL-8 and GROα which are secreted at the basolateral surface of infected cells. This study used a mixture of bovine TLR-transfected HEK293 cells and TLR-DN transfected bovine intestinal epithelial cells 105. Other studies investigating the production of chemokines during *Cryptosporidium* infection also found that IL-8 and GROα were produced in response to infection in HCT-8 and Caco-2 colonic cell lines and human intestinal xenografts in SCID mice models106-108.

Production of antimicrobial peptides (AMP) by the host epithelial cells is triggered during *C. parvum* infection. These peptides include lactoferrin hydrolysate, lactoferricin B, cathelicidin LL37, indolicidin and β-defensins 1 and 2. They were shown to inhibit sporozoite infectivity of *C. parvum* by parasiticidal mechanisms exhibited in Caco-2 cells109. β-defensins have also been found to increase 5- and 10-fold in *C. parvum* infected bovine intestinal epithelial tissue from infected calves, suggesting that they play a key role in epithelial cell defence110. In response, *C. parvum* is able to down-regulate β-defensin gene expression in human HT29 cells, murine CMT-93 cells and BALB/c and C57BL/6 *in vivo* mouse models, although the parasite mechanisms involved are unknown111.

*Cryptosporidium spp.* express a family of secreted proteins known as MEDLE, named after its conserved sequence motif at the C-terminus. They are known to be involved in host cell invasion but their presence and expression varies between *Cryptosporidium* species implying that they may have a role in defining host range. Although the precise function of this protein is still unclear, it gives an insight into how different *Cryptosporidium* species are able to infect different host species112-114. Interestingly, with the establishment of species-specific enteroid models, and methods for creating transgenic parasites, it may be possible to determine how the MEDLE proteins determine host range.

Existing studies have employed immortalised cell lines, including Caco-2, HCT-8 and HT-29 derived from human colon cells and CMT-93 from murine rectal cells, to provide a useful insight into *Cryptosporidium* interactions. None of these cells originate directly from the small intestine and are differentiated into enterocyte-like cells through inducement, which mean they cannot fully recapitulate the properties of the *in vivo* situation. Non-cancerous cell models used to study *Cryptosporidium* include IPEC-J2 derived from porcine jejunal cells115. All of these cell lines are able to form a polarised differentiated monolayer with a brush border, but differ in their secretion and composition of mucous, and their ion permeability due to variation in tight junction assembly82,84,115-118. Although, these cell lines can perpetuate some *in vivo* characteristics, they are not physiologically comparable to the native ileal epithelial cells that *Cryptosporidium* *parvum* would normally invade and so cannot provide an accurate representation of naturally occurring Cryptosporidiosis caused by this common species.

**Use of enteroid models to dissect *Cryptosporidium*-host epithelium interactions**

The use of enteroid models in the study of host-pathogen interactions in *Cryptosporidium* infection is fairly new, and so there are only a few studies to date that make use of these systems. As previously described in *T. gondii*, there are multiple techniques for infection of the apical epithelial surface of enteroids with *Cryptosporidium*, such as fragmentation, microinjection, and production of monolayers. Now, several groups have succeeded in completing the *Cryptosporidium* lifecycle in enteroid culture, generating infectious oocysts for research purposes, and gaining novel biological insight into epithelial-autonomous host defence.

Both 3D human small intestinal organoids and an Air-Liquid Interface (ALI) murine enteroid monolayer model, allow the sustainable generation of new infectious oocysts able to infect mice21,24. The human small intestinal organoids were infected via microinjection and infection was maintained for 28 days before a decline of oocysts was noted. RNA sequencing was also performed to examine the transcriptome of the epithelium during *C. parvum* infection. The data shows that *C. parvum* infection results in altered gene expression related to the Type I IFN pathway. The ALI model was infected by adding calf-derived oocysts directly to the monolayer, which provides easy access to the apical surface of the epithelium, and the infection was maintained for 20 days. Also described are human enteroid monolayers to study aspects of *Cryptosporidium* replication and pathophysiology that have not been fully assessed before19. These novel models are important as they adhere closely to the principles of the 3Rs, with the potential to replace infected calves as the most widely used method of manufacturing infectious oocysts for research purposes, as well as providing the cellular diversity and polarity required to study interactions in a species specific, physiologically relevant model.

As discussed previously, more severe disease, and greater shedding of oocysts, is observed in young children and neonatal calves, declining with age. It may be reasonable to expect that this decline in susceptibility is underpinned by development of adaptive immune responses and immunological memory. However, there is evidence that maturation of the intestinal epithelium itself is a major contributor to age-related resistance. This is supported by enteroid models, which reveal that enteroids derived from neonatal mice support higher quantities of *C. parvum* parasites than enteroids derived from adult mice25.

*Cryptosporidium* causes severe diarrheal disease as it disrupts epithelial barrier function. It does this by increasing the permeability of the epithelium by reducing the levels of tight junction (TJ) proteins, occludin and claudin-4, adherens junction (AJ) protein, e-cadherin and AJ-associated protein, α-catenin. These proteins are components of the epithelial junctional complex which dictates the selective permeability of the epithelium, allowing water, ions and other molecules through, whilst preventing the entry of pathogens and their toxins. Occludin, claudin-4, and e-cadherin were all shown to be down-regulated during *C. parvum* infection in Caco-2 transwell monolayers, murine enteroid monolayers and in the ileum and jejunum of C57BL/6 mice demonstrating that these junctional proteins can be altered by *Cryptosporidium* to induce the diseased state22.

*In vivo* infection with *Cryptosporidium* results in villous atrophy, and enteroid models have been used recently to determine how the parasite affects epithelial growth. Infection of enteroids resulted in reduced crypt budding, and therefore reduced propagation of enteroids. Related to this, decreased expression of stem cell markers, LGR5+ and SOX9, was observed, together with altered expression of genes related to Wnt/β-catenin signalling, which supports stem cell function. Normal cell turnover takes around 3-5 days, but diminished stem cell function slows and even prohibits cell turnover and differentiation. This provides an obvious advantage to parasite propagation as the intracellular phase requires several days to complete its lifecycle within the enterocytes and inhibition of stem cell function facilitates this25.

SLC26A3 (downregulated in adenoma (DRA)) is dysregulated by *Cryptosporidium* infection. This intestinal apical membrane Cl-/HCO3 exchanger protein is involved in chloride absorption in the small intestine, therefore the downregulation of this protein is a major factor in the pathology of *C. parvum*-induced diarrhoea. Although diarrheal disease is usually associated with the disruption of ion transport, DRA dysregulation in *Cryptosporidium* infection has not been described until recently. DRA was shown to be downregulated in Caco-2 cells, and then confirmed in the physiologically relevant models, murine enteroid derived monolayers and *in vivo* in C57BL/6 mice23.

*In vitro* research of *Cryptosporidium* has barely scratched the surface of what is still a poorly understood micro-organism. With the continued application of enteroid models, more accurate investigation of the molecular mechanisms involved in *Cryptosporidium* infection will be possible. In particular, the recent development of bovine enteroids will enable the study of new prophylactic regimes for neonatal calves17,45.

**Use of intestinal organoids to model helminth, bacterial and viral infections**

The enteroid model has not only been used to gain a better understanding of apicomplexan parasites during infection of the intestinal epithelium but they have also been used to investigate helminth infections including *Tritrichomonas muris*, *Heligmosomoides polygyrus*, *Nippostrongylus brasiliensis*, *Trichuris muris*, *Ascaris suum*, and *Trichinella spiralis*119-126. *A. suum* infection has been used in a study to evaluate both the absorptive and barrier functionality of canine intestinal organoids121. Enteroids have also been used to show *T. spiralis* muscle larva and adult worm extracts activating signalling pathways in intestinal tuft cells to initiate type 2 immunity122. The organoid model has allowed for the characterisation of proteins and extracellular vesicles (EVs) secreted by *T. muris* and evaluated their importance in host-parasite communication120. Intestinal organoids have also provided insight into the therapeutic application of helminth EVs such as *N. brasiliensi* EVs in the suppression of colitis and potentially other inflammatory bowel diseases119. A few studies have used organoids as a tool to demonstrate the expansion of tuft cells exposed to IL-13 which is stimulated by IL-25 produced by innate lymphoid cells (ILC) in response to exposure to different nematodes such as *T. spiralis*, *N.brasiliensis, H. polygyrus* and *Tritrichomonas muris*124-126. Enteroids are also currently used as an infection model to study viruses and bacteria. Viral studies using the enteroid model include rotavirus, enteric adenovirus, and norovirus127-131. Bacterial studies suitable for the use of small intestinal enteroids include *Escherichia coli*, *Salmonella*, *Vibrio cholerae*, *Shigella*, and *Clostridium difficile8,132-136*. The use of the enteroid model has also been concentrated on drug development assays and cancer models137-140.

**Future Research**

The use of enteroids not only alleviates the financial and ethical difficulties associated with *in vivo* models but also provides a continuous stream of material to study enteric infection. Regardless of their many advantages, enteroid models still have their limitations. The high cost of setting up these cultures is one aspect that limits their use but hopefully this will be reduced as their use becomes more mainstream in research. With no easy access to the lumen, the lack of an immune system and absent interaction with other body systems, enteroids still require some improvement to overcome these deficiencies56.

Fortunately, these models are currently undergoing development which promises to correct some of the problematic aspects. Transforming 3D enteroids into 2D open format monolayers appears to be the next step in the evolution of these models for ease of access to the apical surface of the epithelium for infection studies14. Human and murine enteroids are fairly well established, but recently livestock enteroids derived from bovine and porcine intestinal tissue have been developed providing a model to study poorly understood livestock infections such as *Cryptosporidium parvum* and *Neospora caninum*17. The hope is to eventually combine these systems together with host specific immune cells, commensal bacteria, and a physiological oxygen gradient so as to further mimic the *in vivo* environment. For example, co-culture of enteroids with dendritic cells will allow us to determine how early epithelial responses dictate the quality of the immune response, and how dendritic cells improve epithelial resistance to infection (Figure 2). Also, in development, is a human enteroid-anaerobe co-culture system that accurately recapitulates the *in vivo* oxygen gradient across the epithelium. The model is able to simulate the presence of the gut microbiota and could be adapted to assess the effect of various components of the microbiota on susceptibility to infection98. Another potential future direction for parasite culture is organ-on-a-chip technology which frequently utilizes Caco-2 cells but could be combined with enteroids to provide a more accessible model to facilitate high throughput experiments to overcome the challenges with culturing *T. gondii* and *C. parvum* sexual stages84.

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**Table 1. Enteroid models used to study *T. gondii* infection.**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Organoid species** | **Infection method** | ***T. gondii* species** | **Stage of lifecycle used** | **Findings of the study** | **Reference** |
| Porcine | * Enteroid

fragmentation | * RH (Type I)
 | Tachyzoites | Demonstrated that organoids could act as a model for enteric *T. gondii* infections. | Derricott *et al.,* 2018 |
| Murine  | * Enteroid fragmentation
* Microinjection
* Epithelial sheets
 | * RH (Type I)
* PRU (Type II)
* VEG strains (Type III)
 | Tachyzoite | Optimized and validated three enteroid based infection techniques. Suggested a role for *de novo* synthesis of cholesterol by the epithelial cells for parasite replication. | Luu *et al.,* 2019 |
| Murine | * Enteroid fragmentation
 | * RH (Type I)
 | Tachyzoites | Demonstration that *T. gondii* infects organoids effectively when the lumen becomes accessible  | Betancount *et al.,* 2019 |
| Feline/ Murine | * Epithelial sheets
 | * ME49 (Type II)
 | Bradyzoite | ﻿Defined the mechanism of species specificity for *T. gondii* sexual development  | Martorelli Di Genova *et al.,* 2019 |

**Table 2.** **Recent *Cryptosporidium* models for the generation of infectious oocysts.**

|  |  |  |  |
| --- | --- | --- | --- |
| Model | Media | Time Maintained | Reference |
| HCT-8 cells in Hollow Fibre Bioreactors (HFB) | MEM with l-glutamine and phenol red, 0.058 g heparin, 0.29 g l-glutamine, 23.8 g HEPES pH 7.8 with 5 M NaOH, 4.5 g d-glucose, 0.035 g ascorbic acid, 0.04 g p-aminobenzoic acid, 0.02 g Ca pantothenate, 0.001 g folic acid, and 100 mL horse serum.  | Over 2 years  | Morada and Yarlett et al., 2016   |
| Caco-2 and HT29-MTX in 3D porous silk scaffolds  | DMEM supplemented with 10% FBS, 10µg/ml human transferrin, 100 U/ml penicillin, 100µg/ml streptomycin, and 0.25 µg/ml amphotericin B  | 15-17 days  | DeCicco RePass et al., 2017  |
| COLO680N cells | 90% RPMI 1640, 10% heat inactivated FBS | 8 weeks | Miller et al., 2018 |
| Human intestinal organoids  | Wnt-CM, Advanced DMEM/F12 with 1 x Glutamax, 10mM HEPES, penicillin-streptomycin, 1 x B27, 1μM N-Acetylcysteine, 20% R-spondin1 conditioned medium, 10% Noggin conditioned medium, 50ng/mL human EGF, 500nM A83-01, 10nM Gastrin, 50% Wnt3a conditioned medium, 10mM nicotinamide, 10μM SB202190, 10nM prostaglandin E2 and 10μM Y-27632.  | 28 days  | Heo et al., 2018  |
| ALI Murine enteroid monolayers  | 50% L-WRN conditioned medium (CM), 10 μM Y-27632 ROCK inhibitor  | 20 days  | Wilke et al., 2019  |
| Human enteroid monolayers  | 50% L-WNT3a-conditioned media, 20% R-Spondin-conditioned media, 10% Noggin-conditioned media, 1 x B27, 1 x N2, 1mM N-acetylcysteine, 50ng/mL Mouse recombinant EGF, 10nM [Leu15]-Gastrin I, 10mM Nicotinamide, 500nM A-83-01 and 10μM SB202190.  | 3 days  | Cardenas et al., 2020  |

**Figure 1. Enteroid models for studying parasitic infections.** (A) Illustration depicting the different cell types, their location, and structures present in an enteroid model. (B-D) Bovine (B), porcine (C) and murine (D) enteroids are depicted showing extensive crypt budding and villus domains on day 4 after passage for bovine and porcine enteroids and day 7 after passage for murine enteroids. (E) Image depicts development of 2D bovine enteroid monolayers on 2mg/mL collagen gels on day 2, 4 and 7 after seeding. The area of the epithelial sheet increases over this period establishing the monolayer by day 7, with an apical surface open to infection. (F) Bovine epithelial sheets maintain some 3D structures (dense red) as well as a population of enteroendocrine cells (green).

**Figure 2: Dynamic imaging of interactions between dendritic cells and *T. gondii* infected intestinal epithelium.** ROSAmT/mG enteroids were infected with *T. gondii* Pru-tdTom-Cre38,141, co-cultured with CFSE-labelled “gut-like” bone marrow-derived dendritic cells, and imaged by 2-photon microscopy. The images depict four time points from a time-lapse movie. Enteroid epithelial cells from ROSAmT/mG mice express membrane tdTomato (mT; red outlines), until exposure to Cre-recombinase, when they begin to express membrane eGFP (mG; green). Pru-tdTom-cre parasites (solid red) secrete cre into the host cell upon invasion, turning infected epithelial cells green. Dendritic cells (cyan) are seen interacting with the basal surface of the enteroid.