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SUMMARY

Cryptosporidium is a major cause of moderate to severe diarrhea in humans worldwide, second only to rotavirus. Due to the wide host range and environmental persistence of this parasite, cryptosporidiosis can be zoonotic and associated with foodborne and waterborne outbreaks. Currently, 31 species are recognized as valid and of these, Cryptosporidium hominis and Cryptosporidium parvum are responsible for the majority of infections in humans. The immune status of the host, both innate and adaptive immunity, has a major impact on the severity of the disease and its prognosis. Immunocompetent individuals

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typically experience self-limiting diarrhea and transient gastroenteritis lasting up to 2 weeks and recover without treatment, suggesting an efficient host anti-parasite immune response. Immunocompromised individuals can suffer from intractable diarrhea, which can be fatal. Effective drug treatments and vaccines are not yet available. As a result of this, the close cooperation and interaction between veterinarians, health physicians, environmental managers and public health operators is essential to properly control this disease. This review focuses on a One Health approach to prophylaxis, including the importance of understanding transmission routes for zoonotic Cryptosporidium species, improved sanitation and better risk management, improved detection, diagnosis and treatment and the prospect of an effective anti-cryptosporidial vaccine.

**Keywords:** Cryptosporidium, diagnosis, treatment, zoonotic, risk management, vaccines, prophylaxis

**INTRODUCTION**

*Cryptosporidium* species are protozoan parasites that infect a broad range of hosts including humans, domestic and wild animals worldwide, causing asymptomatic or mild to severe gastrointestinal disease in their host species (1-6). Currently, 31 *Cryptosporidium* species have been recognised as valid and of these, by far the most common species reported in humans worldwide are *C. parvum* and *C. hominis* (7-12).

Human cryptosporidiosis is frequently accompanied by abdominal pain, fever, vomiting, malabsorption and diarrhea that may sometimes be profuse and prolonged (13, 14). The immune status of the host, both innate and adaptive immunity, has a major impact on the severity of the disease and its prognosis. Immunocompetent individuals typically experience self-limiting diarrhea and transient gastroenteritis lasting up to 2 weeks and recover without
treatment, suggesting an efficient host anti-parasite immune response. Immunocompromised individuals including HIV/AIDS patients (not treated with antiretroviral therapy), often suffer from intractable diarrhea, which can be fatal (15). An effective vaccine for cryptosporidiosis is not yet available.

The Global Enteric Multicenter Study (GEMS) study, which was a three year matched case-control study of moderate-to-severe diarrhea in over 22,000 infants and children at seven sites across Africa and Asia aged 0-59 months, found that Cryptosporidium was second only to rotavirus as a major cause of severe diarrhea (16, 17). More recent matched case-control studies of diarrhea have confirmed this (18). Similarly, a birth cohort study conducted by a Global Network for the Study of Malnutrition and Enteric Diseases (MAL-ED), has assessed pathogen-specific burdens in diarrheal and nondiarrheal stool specimens from 2,145 children aged 0-24 months, over five years at eight community sites in Africa, Asia and South America, and identified Cryptosporidium spp. as one of the five highest attributable burdens of diarrhoea in the first year of life (19). Globally, cryptosporidiosis is estimated to be responsible for the majority of deaths among children under 5 years of age (20-22) and Cryptosporidium infection in children is also associated with malnutrition, persistent growth retardation, impaired immune response and cognitive deficits (23, 24). The mechanism by which Cryptosporidium affects child growth seems to be associated with inflammatory damage to the small intestine (25). Undernutrition (particularly in children) is both a sequela of- and a risk factor for- cryptosporidiosis, particularly in low-income countries (26-31). FAO’s executive summary of the State of Food Insecurity in the World (http://www.fao.org/docrep/018/i3458e/i3458e.pdf) indicates there are 842 million chronically malnourished persons worldwide, which significantly contributes to impaired immunity and thus increased susceptibility to infection with Cryptosporidium, perpetuating
the cycle of chronic diarrhea and malnutrition. In developed countries, Cryptosporidium is less common (15) and accounts for ~9% of diarrheal episodes in children (32).

Cryptosporidiosis is a highly prevalent and extremely widespread disease (6) and several factors contribute to this. Infected individuals shed large numbers of oocysts, which are environmentally very robust, resistant to inactivation by commonly used drinking water disinfectants including chlorine treatment and are able to survive routine wastewater treatments (33, 34). Cryptosporidium oocysts are highly infectious; in human volunteer studies, as few as 10 or less Cryptosporidium oocysts can produce disease in healthy adults (35, 36). A quantitative risk assessment has estimated that ingestion of a single oocyst of the C. parvum IOWA isolate will result in clinical disease in 2.79% of immunologically normal persons (37). Another contributing factor to the high prevalence and widespread distribution of Cryptosporidium is the lack of treatment options. Only one drug, nitazoxanide (NTZ, Alinia; Romark Laboratories, Tampa, Florida, United States), has been approved by the United States (US) Food and Drug Administration (FDA). This drug, however, exhibits only moderate clinical efficacy in malnourished children and immunocompetent people, and none in immunocompromised individuals like people with HIV (38, 39).

Because oocysts of Cryptosporidium species from humans and animals are ubiquitous in the environment, cryptosporidiosis can be acquired through multiple routes (reviewed by Robertson et al. 40). Transmission of oocysts is by the faecal-oral route, either directly or indirectly. For humans, direct transmission can be from person to person primarily due to poor hygiene among household members and attendees in day-care centers, aged care facilities and other institutions, or from animals to persons such as farm-workers and pet owners. Most indirect transmission is from contaminated drinking or recreational water. Contaminated food can also be a source of transmission and contamination can occur at every step throughout the food preparation process, from farm to table (41, 42). Findings from
animal models, human case reports, and a few epidemiological studies suggest that Cryptosporidium may also be transmitted via inhalation of aerosolized droplets or by contact with fomites contaminated by coughing (see 43).

The “One Health” approach to tackle zoonotic diseases defined as "One Medicine" by Schwabe (1984), is a worldwide strategy to improve health and well-being through the mitigation and prevention of disease risks that originate at the interface between humans, animals and their various environments (44). This review focuses on a One Health approach to prophylactic prevention of cryptosporidiosis including improved detection, diagnosis and treatment, the importance of understanding zoonotic transmission, better environmental and risk management and the prospect of an effective anti-cryptosporidial vaccine.

**DETECTION, DIAGNOSIS AND TREATMENT**

Cryptosporidium presents many challenges for detection and diagnosis. The use of different diagnostic methods and the inconsistent application of typing techniques can make direct comparisons difficult or even impossible between clinical, veterinary and environmental testing or between different regions or countries (45). Detection of Cryptosporidium in clinical pathology laboratories is still based mainly on microscopic detection via stains and/or fluorescent antibodies (IFA) and other antigenic detection methods. Although microscopy needs relatively simple instruments and cheap consumables, it is labour intensive, requires a skilled operator and lacks sensitivity and specificity (45). Morphological characters for identifying Cryptosporidium are few (46, 47) and differential staining techniques are usually required due to the fact that oocysts are similar in size and shape to yeasts, fecal components and other debris (47, 48). Acid fast (AF) modified Ziehl–Neelsen staining is one of the most common differential staining techniques (45, 48). However, the detection limits of
conventional microscopy for *Cryptosporidium* have been reported to be as low as 50,000 to 500,000 oocysts per gram of human faeces (49), resulting in low levels of infection or sporadic shedding possibly going unnoticed when conventional methods of detection are used. Sporadic shedding is such that studies have shown that three separate faecal samples should be examined for immunocompetent patients and two samples for AIDS patients for confident diagnosis of cryptosporidial infections using acid-fast staining (50). IFA stains offer superior sensitivity; in some studies, about 97% sensitivity compared with only about 75% sensitivity for acid-fast staining (51). However, IFA is more expensive than acid-fast staining and requires a fluorescence microscope and trained staff (51). This is particularly problematic in resource-poor areas where cryptosporidiosis is a major health problem. A recent study proposed the use of phase contrast microscopy (PCM) as a specific and inexpensive method for detection of *Cryptosporidium*, however this method still lacks sensitivity (52).

Other antigen detection formats such as enzyme linked immunosorbent assays (ELISA’s), enzyme immunoassays (EIA’s) and immunochromatographic (dipstick) assay for *Cryptosporidium* are also commercially available and have the advantage of reducing assay times and being amenable to automation. However, diagnostic sensitivities are variable (70% to 100%) (51, 53-55); some rapid tests have reduced specificity and sensitivity for species other than *C. parvum* or *C. hominis* (56, 57) and confirmation of positive reactions is needed (55). Biosensor chips, that detect and quantitate *C. parvum* in real-time via anti-*C. parvum* IgM binding have also been developed (58, 59), however detection limits are relatively high (100 or more oocysts) and they have yet to be fully evaluated on water or faecal samples. Another major limitation of both conventional microscopy and antigen detection methods is that they cannot identify to species or subtype level, which is essential for understanding transmission dynamics and outbreaks, in particular for zoonotic species.
Polymerase chain reaction (PCR)-based techniques have permitted specific and sensitive detection and differentiation of Cryptosporidium spp. for clinical diagnosis and environmental monitoring (45). Real-time or quantitative PCR (qPCR) assays have been developed to quantitate the numbers of Cryptosporidium oocysts present in human and animal faecal and water samples (60-63) with 100% specificity and sensitivities as low as 200 oocysts per gram of faeces, which equates to 2 oocysts per PCR (60). Multiplex qPCR assays have also been developed for the detection of Cryptosporidium and other common causes of diarrhoea such as Giardia duodenalis and Entamoeba histolytica, which have the advantage of identifying mixed infections (64-66). The most widely used molecular markers for identification and typing of Cryptosporidium species are the 18S ribosomal RNA (18S rRNA) gene and the 60-kDa glycoprotein (gp60) gene respectively (4, 10). Miniaturised fluidic devices, which can detect to species level have also been developed, mainly for the water industry, (reviewed by Bridle et al. 67), but as with antibody-based biosensor chips, have yet to be fully validated and are costly.

New drug targets for Cryptosporidium are urgently needed, as the only FDA-approved drug, nitazoxanide, does not provide benefit for malnourished children and immunocompromised patients with cryptosporidiosis. However, Cryptosporidium has completely lost the plastid-derived apicoplast present in many other apicomplexans, and the remnant mitochondrion lacks the citrate cycle and cytochrome-based respiratory chain (68). Therefore, many classic drug targets are unavailable in Cryptosporidium. Progress in developing anti-cryptosporidial drugs has also been affected by the inability to generate large numbers of Cryptosporidium oocysts in vitro and an inability to genetically manipulate the organism (69, 70). The recent development of a hollow fiber in vitro culture system to generate large numbers of oocysts (up to $10^8$ oocysts per day) (71) and advances in
genetically engineering Cryptosporidium (72), will transform the development of novel therapeutics.

To date, the best studied drug target is the bacterial derived inosine 5’- monophosphate dehydrogenase (IMPDH) gene, as Cryptosporidium does not contain guanine salvage enzymes and is totally dependant on this enzyme to convert adenosine salvaged from the host into guanine nucleotides (69, 74-76). This coupled with the parasite’s high metabolic demand for nucleotides due to the complicated lifecycle of this parasite make IMPDH an important drug target (77-85).

Other drug targets include long chain fatty acyl-coenzyme A synthetases (LC-ACS) which are essential in fatty acid metabolism (68) and a recent study reported good efficacy of the ACS inhibitor triacsin C against cryptosporidial infection in mice (86). A parasite cysteine protease inhibitor was also effective in vitro and in an animal model (87). Other studies have focused on repurposing existing drugs to overcome the prohibitive costs of de novo drug development (estimated to be between $500 million and $2 billion per compound successfully brought to market) (88). For example, several compounds from the Medicines for Malaria Venture (MMV) Open Access Malaria Box have exhibited activity against C. parvum (89) and drugs such as the human 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitor, itavastatin and Auranofin (Ridaura®) initially approved for the treatment of rheumatoid arthritis and have been shown to be effective against Cryptosporidium in vitro (90, 91), which holds promise for further in vivo testing in animals and humans.
ZOONOTIC CRYPTOSPORIDIUM SPECIES

Due to the morphological similarity of Cryptosporidium oocysts from different host species, initial findings of Cryptosporidium infections in both domestic and wild animals were assumed to be due to C. parvum leading to an overestimation of the potential role of animals as reservoirs of human disease (92). However, with the assistance of advanced molecular techniques, many of these species in wildlife particularly were identified as host-adapted genotypes (6, 10). Of the 31 Cryptosporidium species that have been recognised as valid, more than 20 species and genotypes have been identified in humans including C. hominis, C. parvum, C. meleagridis, C. felis, C. canis, C. cuniculus, C. ubiquitum, C. viatorum, C. muris, C. suis, C. fayeri, C. andersoni, C. bovis, C. scrofarum, C. tyzzeri, C. erinacei and Cryptosporidium horse, skunk and chipmunk I genotypes, with C. hominis and C. parvum most commonly reported (4, 6, 10). These Cryptosporidium spp. infect both immunocompetent and immunocompromised persons (6, 10). Of these, C. parvum and C. hominis are by far the most common species reported in humans worldwide (4, 93) and are responsible for most cryptosporidiosis outbreaks, with C. hominis responsible for more outbreaks than C. parvum in most regions (4). Although humans are the major host species for C. hominis, there have been isolated reports in domestic animals and wildlife hosts including sheep, goats, cattle, a dugong, non-human primates and kangaroos (94-102, Zahedi et al., unpublished) and in fish (103). Cryptosporidium parvum is primarily a parasite of artiodactyls and humans (4) but has also been frequently reported in wildlife, including various rodents, bovids, camelids, equids, canids, non-human primates and marine mammals (see 10) and in fish (103-106). Cryptosporidium meleagris, although primarily a bird parasite, is the third most prevalent species infecting humans (4, 6, 93) and in some studies, C. meleagridis prevalence is similar to that of C. parvum (107, 108). Cryptosporidium cuniculus (previously known as rabbit genotype) was responsible for a drinking-water
associated outbreak of cryptosporidiosis in the UK (109-111) and has also been identified in many sporadic human cases of cryptosporidiosis (112-115). It is also the third most commonly identified Cryptosporidium species in patients with diarrhoea in the UK (112). Human infections with *C. canis* and *C. felis* have been reported mainly in studies conducted in children in developing countries (6) where they are responsible for as much as 3.3 and 4.4%, respectively, of overall cryptosporidiosis cases (116). *Cryptosporidium muris* is also considered a zoonotic species as there have been numerous reports of *C. muris* in humans and one report in human sewage (117-129). In a recent human infectivity study, *C. muris* was examined in six healthy adults (130). Volunteers were challenged with $10^5$ *C. muris* oocysts and monitored for 6 weeks for infection and/or illness. All six patients became infected. Only two of the infected volunteers had a diarrheal illness (a 33% illness attack rate). Three other volunteers passed an occasional unformed stool or typically had a single soft stool per day without any accompanying gastrointestinal symptoms (130). Like *C. muris*, *C. andersoni* is also a gastric parasite and primarily infects the abomasum of cattle and to a lesser extent, sheep and goats (6, 93). It is occasionally detected in humans (cf. 10). Two studies in China by the same research group have reported that *C. andersoni* was the most prevalent *Cryptosporidium* species detected in humans (131-132). However, further research is required to better understand the zoonotic importance of *C. andersoni*.

*Cryptosporidium ubiquitum* is of public health concern because of its wide geographic distribution and broad host range (133). It has been frequently reported from drinking source water and wastewater in various geographic locations, and is considered an emerging zoonotic pathogen as it has been identified in many human cases of cryptosporidiosis (133). Subtyping at the gp60 locus has suggested that sheep and wild rodents are a key source of *C. ubiquitum* transmission to humans, through either direct human contact with infected animals or by contamination of drinking source water (133). It is thought that human encroachment
into wildlife territories has been responsible for the emergence of *C. ubiquitum* and other genotypes like chipmonk genotype I and to a lesser extent, skunk and mink genotypes in human populations (133-143). This highlights the importance of extensive molecular epidemiological studies of wildlife to better understand the public health risks.

**RISK MANAGEMENT**

A key part of a One Health approach to *Cryptosporidium* prophylaxis is a better understanding of environmental, epidemiological and aetiological factors associated with cryptosporidial infections to enable more targeted risk management. The far-reaching One Health strategy aims at integrating multidisciplinary knowledge and evidence, and at coordinating the interventions, in order to create a global synergism catering for all aspects of health-care for humans, animals and the environment (the One Health Triad).

Under an environmental perspective, the prophylaxis of waterborne cryptosporidiosis must consider optimal management (or design) of source, recycled– and recreational– waters. Protection of source water and swimming pools is a key element of *Cryptosporidium* prevention as contamination of drinking water and swimming pools is a major mode of transmission (see ref 33, 144, 145) and is often achieved by restricting the access to catchments and water bodies, while swimming pools are designed and monitored according to construction standards and guidelines. Infection prevention and management however, can only be achieved through a deep understanding of the routes of transmission, sources of contamination (human and animal), disease prevalence in the population, and the risk factors in the final host.

The link between *Cryptosporidium* in drinking water and sporadic infections is well documented (33, 144, 145), however, the association between drinking water contamination and endemic cryptosporidiosis is not well established. For example, some studies reporting
drinking unsafe water as a risk factor for endemic cryptosporidial infection (146, 147) and others report no association (148-150). Seasonal patterns are also thought to be associated with an increased transmission risk (151, 152), such as when recreational waters are more heavily utilised.

High precipitation events favor the transfer and survival of oocysts in surface waters and/or groundwater (153, 154). This may result in contamination of source water and increased risk of cryptosporidiosis depending on the source of contamination (154). Indeed, the average odds of identifying *Cryptosporidium* oocysts in fresh surface waters is 2.61 (95% CI = 1.63-4.21; I² = 16%) times higher during and after extreme weather events (155). Shifts in precipitation patterns (intensity and location) is one of the climate change predictions for the future (156) and this will clearly impact both waterborne and foodborne transmission of *Cryptosporidium* and therefore future human exposures may differ significantly from current patterns as the climate changes (157). Hydrodynamic modelling has been shown to represent a valid and cost-effective support, for decision making and understanding of events (158). Quantitative microbial risk assessment (QMRA), is another widely used tool to estimate health impacts from exposure to *Cryptosporidium* and other pathogens (155) and has been applied to climate change (159). The tool, called CC-QMRA (Climate Change Quantitative Microbial Risk Assessment), quantifies the anticipated impacts in terms of relative infection risks under climate change scenarios for *Cryptosporidium* and other pathogens and can be used to evaluate impacts of climate change on infection risks from waterborne and foodborne transmission of *Cryptosporidium* (160). For example, CC-QMRA can be used to evaluate the effectiveness of interventions such as upgrading wastewater and drinking water treatment and strengthening drinking water and bathing water regulations.
Quantitatification and identification of *Cryptosporidium* in wildlife excreta is an essential starting point for estimating catchment loads (161). Variables such as soil physicochemical properties, hydrology, orography, meteorology etc. can all affect oocyst viability, transport and fate. Source water contamination can be avoided or reduced by the implementation of management strategies like wildlife population control, revegetation, landscaping and soil conditioning. In addition, effective risk management cannot overlook the prevalence, infectivity and zoonotic potential of *Cryptosporidium* isolates in the animal populations within the catchment. Similarly, recreational waters like swimming pools, sauna, spas, aquatic parks etc. are also potential sources of outbreaks, depending on the age and health status of the users (and maintenance). Personal hygiene practices (e.g. showering before swimming in public swimming pools, washing hands before cooking, eating and after defecation and washing fruits and vegetables before consumption), are an essential part of any prevention strategy and can prevent/reduce disease transmission (162-165). Enforcing and encouraging similar practices, however, becomes absolutely crucial during outbreaks and in the presence of hyper-susceptible final hosts.

It has been shown that an important host risk factor includes HIV status. *Cryptosporidium* is an important pathogen regardless of HIV-prevalence (16), however HIV-positive children are between 3 and 18 times more likely to have *Cryptosporidium* than those who were HIV-negative (166-168). With the widespread availability of antiretroviral therapy, particularly in industrialised countries, the incidence of cryptosporidiosis has decreased among people living with AIDS (169). However, the increasing number of transplant recipients and those receiving immunosuppressive drugs may contribute significantly to the burden in the future (170, 171). Malnutrition is also a risk factor for both diarrhea and prolonged diarrhea caused by *Cryptosporidium*, with significantly higher rates of infection in malnourished children controlling for HIV status (172-175). An unknown number of
individuals experience asymptomatic Cryptosporidium infection (176). This clinically silent infection may remain undetected and untreated and therefore may contribute not only to parasite transmission but also to malnutrition and the associated clinical sequelae. Breastfeeding may provide some protection, as a recent study of Bangladeshi infants reported that protection from Cryptosporidium infection was associated with high anti-Cryptosporidium IgA in breast milk (177).

VACCINES

The development of vaccines for cryptosporidiosis, particularly in vulnerable populations such as children and malnourished populations is urgent, but has been hampered by an incomplete understanding of the host immune response to Cryptosporidium (178, 179). Therefore, a better understanding of host-parasite interactions is crucial for the development of an effective vaccine (178). Given that adults in highly endemic areas are partly immune to reinfection; and human challenge studies have shown that previous infection or exposure leads to a higher infectious dose [ID<sub>50</sub>] (180, 181), development of a successful vaccine should be possible. It is known that both innate and adaptive host response are important in the control of Cryptosporidium infection (182-184). Yet the nature of these responses, particularly in humans, is not completely understood (179, 185).

Early mediators of innate immune protection include the thick mucus layer of the small intestine, intestinal epithelial cells and chemokines, cytokines and antimicrobial peptides secreted into the intestinal lumen and/or underlying submucosa and bloodstream (179). Important cytokines include interferon gamma (IFN-γ), which is secreted early in infection by natural killer (NK) cells, macrophages and dendritic cells which are thought to play a major role in orchestrating both the innate and adaptive immune responses (179, 183). Th1 inflammatory response, cytokines, such as interleukin 12, 15 and 18 are also important in the
resistance and recovery to Cryptosporidium infection (186-191). Treatment of both immunocompetent and immunodeficient mice with IL-12 before infection prevented or greatly reduced the severity of infection and was attributed to a decrease in IFN-γ reduction (191). Data suggests that IL-15 has an important role in activating an NK cell-mediated pathway that leads to the elimination of Cryptosporidium from the intestine (187). IL-18 is produced by epithelial cells in the gut and a number of different immune cells and is upregulated in response to C. parvum infection and it has been proposed that one of the functions of IL-18 is to promote IFN-γ expression by macrophages (188). Toll-like receptors expressed by epithelial cells have been shown to be important in modulation of the host immune response and subsequent parasite clearance (192-198).

MicroRNA (miRNA) regulation also appears to play an important role in host cell protection against Cryptosporidium (199-204). miRNA are small RNA molecules of 23 nucleotides that result in gene silencing via translational suppression or mRNA degradation and are a mechanism to fine-tune cellular responses to the environment, and may be regulators of host anti-microbial immune responses (202). More than 700 miRNAs have been identified in humans and are postulated to control 20-30% of human genes. miRNA-mediated post-transcriptional gene regulation may regulate expression of genes critical to epithelial anti-microbial defense and one cellular miRNA (let-7i) has been shown to target Toll-like receptor 4 (TLR4) and regulate TLR4-mediated anti-C. parvum defense (199). Functional manipulation of select miRNA expression levels in epithelial cells has been shown to alter C. parvum infection burden in vitro (202, 203). The intercellular adhesion molecule-1 (ICAM-1; CD54) is a 90 kDa member of the Ig superfamily expressed by several cell types including endothelial cells and epithelial cells and is thought to facilitate adhesion and recognition of lymphocytes at infection sites as ICAM-1 is constitutively present on endothelial cells and epithelial cells, but its expression is increased by pro-inflammatory cytokines or following
microbe infection. Evidence has shown that miR-221-mediated translational suppression controls ICAM-1 expression through targeting the ICAM-1 3′-untranslated region (UTR), in epithelial cells in response to C. parvum infection, as transfection of an miR-221 precursor in an vitro model of human biliary cryptosporidiosis abolished C. parvum-stimulated ICAM-1 protein expression (202).

Mannose-binding lectin (MBL) is an evolutionarily conserved protein, secreted by hepatocytes, that functions in human innate immunity by binding to microbial surfaces and promoting opsonophagocytosis. MBL has been shown to be important in the protection against cryptosporidiosis, as children and HIV-infected adults with mannose-binding lectin deficiency have increased susceptibility to cryptosporidiosis and more severe disease (205-207). The genetic contribution to deficient or low serum levels of MBL results from polymorphisms in the MBL2 gene (MBL1 is a pseudogene), which create low MBL-producing MBL2 genotypes in ~5% of the world’s population (207). In one study on a cohort of preschool children from Dhaka, Bangladesh, polymorphisms in the MBL2 gene (and corresponding haplotypes) and deficient serum levels of MBL were associated with increased susceptibility to infection with Cryptosporidium. MBL deficiency of <500 ng/mL was associated with single and multiple symptomatic episodes of Cryptosporidium infection, with an OR of 7.6 for children with multiple symptomatic infections with Cryptosporidium (207). The mechanism by which MBL controls Cryptosporidium infection and protects children from it is still not clearly understood.

Adaptive immunity creates immunological memory after an initial response to Cryptosporidium and leads to an enhanced response to subsequent encounters with Cryptosporidium. For example, antibodies to the parasite antigen gp15 were associated with protection against reinfection (208). The adaptive immune response to Cryptosporidium is characterized as a T-helper 1 (Th1) response (189) and the importance of the adaptive
immune response during *Cryptosporidium* infection is highlighted by the susceptibility of AIDS patients to cryptosporidiosis, as well as the resolution of infection observed following CD4+ T lymphocyte cell reconstitution in patients given antiretroviral therapy (185, 209). Low absolute CD4+ T cell counts in patients with HIV/AIDS were thought to be responsible for persistent and severe cryptosporidiosis, however research with Simian immunodeficiency virus (SIV)-infected macaques reported that persistent cryptosporidiosis was more dependant on SIV load and profound viral damage to gut lymphoid tissue and rapid depletion of mucosal CD4+ T cells during the acute phase of viral infection, than on declining circulating CD4+ T cell levels during chronic SIV infection (210). This suggests that depletion of local CD4+ T cells may be more predictive of disease severity than absolute CD4+ T cell numbers. The importance of other T cells such as CD8+ have not been extensively studied but do appear to play a role (179). The role of humoral immunity in protection from cryptosporidiosis is not well understood and no clear surrogate marker of protective immunity exists (reviewed in 46 and 179).

The ideal *Cryptosporidium* vaccine should provide rapid life-long immunity in all vaccinated individuals, be broadly protective against the most common species and subtypes of *Cryptosporidium*, prevent disease transmission, and be readily accessible, stable, and cheap (178, 179). Ensuring cross-reaction against the most common species infecting humans however will be difficult, as more than 20 *Cryptosporidium* species and genotypes can infect humans as discussed above. For example, a recent study showed that infection of gnotobiotic pigs with *C. hominis* resulted in complete protection against subsequent infection with *C. hominis*, but incomplete protection against infection with *C. parvum* (211), therefore multiple species will need to be targeted to provide sufficient cross-protection. In addition, as children, malnourished and immunocompromised individuals are the most important vaccine targets, they may not be able to develop a strong and sustained immune-mediated protection in

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response to vaccination. Indeed, malnutrition has been cited as an important factor underlying limited efficacy of vaccines (212). It is therefore likely that adjuvants such as TLR ligands (213), will be required to enhance the immune-response in target populations (193, 196).

Several antigens, aimed at raising immunoglobulin G antibodies, are being developed as vaccine candidates (178). Some of the best studied are gp15 (214-219), cp15 (220-225) and cp23 (223, 226, 227). The gp15 antigen is derived from the glycoprotein gp60, which is cleaved by a parasite serine proteinase into two surface proteins - gp15 and gp40, both of which play an essential role in parasite motility and attachment to and invasion of host epithelial cells (228), and can stimulate interferon γ production by peripheral blood mononuclear cells of those previously infected (214). The gp15 antigen is relatively conserved between C. parvum and C. hominis, and studies in Bangladesh indicated that there is significant cross-reactivity between them and that antibodies to gp15 were associated with shorter duration of illness (217). Similarly, in a study in Kenya, AIDS patients without diarrhea had significantly higher serum IgG levels to gp15 than those with diarrhea (229).

cp15 is an immunodominant protein present on the oocyst surface and is associated with internal structures and bears no apparent similarity to gp15 (228). Immunisation of pregnant goats with cp15 vaccines protected off-spring (230). The impact of malnutrition however on vaccination was demonstrated in recent research on intranasal vaccination of nourished and malnourished mice, with the cp15 antigen primed with a live enteric bacterial vector (225). The authors reported that malnutrition blunted antigen-specific cell-mediated responses to cp15 and that vaccination resulted in only transient reduction in stool shedding of Cryptosporidium and was not protective against disease (225).

cp23 is an immunodominant protein, geographically conserved among C. parvum isolates, is present in both the sporozoite and merozoite stages (178), and antibodies to it are frequently detected following Cryptosporidium infection (231, 229). Serum antibodies to
both gp15 and cp23 are associated with protection from diarrhea in immunocompetent adult human volunteers infected with Cryptosporidium (208, 232-234). Thus, a multivalent vaccine, incorporating multiple antigens or antigenic epitopes, may enhance protection against infection. For example a divalent cp23 and cp15 vaccine prolonged the prepatent period and decreased oocyst shedding in mice vaccinated with the divalent vaccine compared with vaccination with cp23 alone (223). Similarly a reverse vaccinology approach based on genome mining that included three antigens; the well characterised cp15, a calcium-activated apyrase involved in the invasion process of Cryptosporidium and profilin, an agonist of the innate immune system through its recognition by Toll-like receptors, induced specific and potent humoral and cellular immune responses in mice, however, further studies are necessary to verify the protection induced by these antigens (224). The development of an effective vaccine against Cryptosporidium is still a challenge and a better understanding of which immune responses are necessary for protection are essential to the development of immune-based interventions.

CONCLUSIONS
Recent developments have improved our understanding of both the genetics of and immune response in cryptosporidial infections. However, many knowledge gaps remain. Current diagnostic tests each have their limitations in cost, performance, differentiation of clinical significance, and assessment of co-infections with other pathogens. Inaccessability of diagnostic testing in non-industrialised has meant that the knowledge of the epidemiology of Cryptosporidium infection in early infancy is scarce, and as a result, the burden of cryptosporidiosis is under-reported and under-estimated, which reinforces ineffective clinical and public health management of Cryptosporidium. Rapid, cost-effective and reliable diagnostic tests therefore need to be developed for non-industrialised countries to improve
detection, reporting and interpretation of results in the setting of multiple infections. With the recent improvements in cell culture and genetic manipulation, identification of novel or repurposed therapeutics should be radically transformed. Vaccines have the potential to reduce the significant burden of disease, but the extent and types of immunity necessary, and the methods by which to administer and induce protective immunity need further study.

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The authors have no potential conflict of interest to disclose.

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