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Cryptosporidium Pathogenicity and Virulence

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SUMMARY

Cryptosporidium is a protozoan parasite of medical and veterinary importance that causes gastroenteritis in a variety of vertebrate hosts. Several studies have reported different degrees of pathogenicity and virulence among *Cryptosporidium* species and isolates of the same species as well as evidence of variation in host susceptibility to infection. The identification and validation of *Cryptosporidium* virulence factors have been hindered by the renowned difficulties pertaining to the *in vitro* culture and genetic manipulation of this parasite. Nevertheless, substantial progress has been made in identifying putative virulence factors for *Cryptosporidium*. This progress has been accelerated since the publication of the *Cryptosporidium parvum* and *C. hominis* genomes, with the characterization of over 25 putative virulence factors identified by using a variety of immunological and molecular techniques and which are proposed to be involved in aspects of host-pathogen interactions from adhesion and locomotion to invasion and proliferation. Progress has also been made in the contribution of host factors that are associated with variations in both the severity and risk of infection. Here we provide a review comprised of the current state of knowledge on *Cryptosporidium* infectivity, pathogenesis, and transmissibility in light of our contemporary understanding of microbial virulence.

INTRODUCTION

Our understanding of infectious diseases has been deeply influenced by the fathers of microbiology, Robert Koch and Louis Pasteur, who independently demonstrated the relationship between the microbial world and disease (1). Their work and rationale led directly to the identification and characterization of the etiological agents of the world's major infectious diseases. Subsequent investigators, however, noted that the causal relationship

between microbes and disease can be less straightforward than originally envisaged by Koch and Pasteur, with a variety of host and pathogen factors affecting the occurrence and outcome of disease, particularly in cases of opportunistic infections associated with a compromised host immune system. In considering this dichotomy, Isenberg reflected in 1988 that while “doubt about the meaning of pathogenicity and virulence seems inappropriate, if not ridiculous,” the traditional vocabulary associated with infectivity and virulence was nevertheless insufficiently well defined to accommodate contemporary observations of clinically important infectious diseases (1). Despite the work of Isenberg and others to clarify and unify a set of appropriate descriptive terminology in this area, some confusion persists, undermining contributions from the major technological advances of our time: genome sequencing, real-time PCR, DNA microarrays, transcriptomics, genetic manipulation, RNA silencing, monoclonal antibodies, fluorescence imaging techniques, and proteomics. This confusion correlates with the fact that the majority of the virulence factors from the microorganisms responsible for the world's most prevalent diseases remain poorly defined and uncharacterized. In this review, we begin by providing a straightforward and generically applicable lexicon for the discussion of pathogenicity and virulence and subsequently focus on the medically important waterborne protozoan parasite *Cryptosporidium* sp., synthesizing modern studies, especially the elucidation of the *Cryptosporidium parvum* and *C. hominis* genomes, which were complemented by

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existing and ongoing research describing the virulence factors which may underpin the molecular mechanisms of *Cryptosporidium* pathogenesis.

CONTEMPORARY DESCRIPTION OF VIRULENCE

Virulence is commonly defined simply as the ability of a microorganism to cause disease (2–9). Virulence and pathogenicity are often used interchangeably, but virulence may also be used to indicate the degree of pathogenicity, where pathogenicity is used solely to describe the ability of a pathogen to inflict damage to the host. Virulence is commonly used to describe the likelihood of an infected person becoming ill as well as the severity of symptoms. Thus, virulence could be considered an increased risk of infection and/or an increased severity of illness, and in light of this, Woolhouse and colleagues (10) defined virulence as, “the direct or indirect reduction in host fitness attributable to pathogen infection.” Pathogen-centered views of virulence assert that pathogens are distinguished from nonpathogens by their expression of virulence factors. Although this concept appears to apply to some microbes that cause disease in healthy hosts, it has been pointed out that this does not apply to microbes that, like *Cryptosporidium*, cause serious disease primarily in immunocompromised hosts (11). While earlier definitions of pathogen-centered pathogenicity and virulence are understandable, the modern consensus is that neither microbe nor host characteristics can independently cause disease (12). Virulence, despite being a microbial characteristic, can exist only in a susceptible host and depends on the context and nature of the host-microbe interaction. Thus, a qualified description of virulence with which we concur can be phrased as a pathogen property which can be modulated by host susceptibility and resistance and which causes damage to the host (12). This description takes into account the complexity of host-pathogen interactions and the contribution of both parties to virulence and uses host damage to define virulence (11).

The view that virulence is a single characteristic, however, is difficult to reconcile with the fact that host-pathogen interactions are continuous and subject to changes in host, microbial, and exogenous factors (11). In fact, virulence is usually multifactorial, involving a complex interplay between the parasite and the host. Various host factors, including age, sex, and the status of the immune system, affect the outcome of the host-parasite interaction (13). In addition, the genotypic and phenotypic characteristics of the parasite define intrinsic diversity in isolate pathogenicity and virulence (14). Therefore, an integrated view of microbial pathogenesis and virulence accounting for the contribution of both host and pathogen factors and accommodating a spectrum of host-pathogen interactions provides a more accurate perspective (10, 12).

Each of the microbiological attributes that contribute to virulence can, in general, be linked to specific structural elements or biochemical compounds within the organism; these are generally termed virulence factors. Although the terms “virulence determinants” and “virulence factors” are widely used to describe traits contributing to pathogenicity, a subtle distinction exists between the two terms. Virulence factors are “microbial traits that promote host damage” (8), and more precisely, a virulence factor is a gene product necessary but not sufficient to cause disease. In this context, virulence factors can be defined as “contributory virulence factors” (11), which are not singular determinants of virulence but are able to influence the severity and duration of the disease; there-

fore, the mutation of such virulence factors should still allow the pathogen to cause disease. This can be illustrated by the significantly reduced (but still present) virulences of a variety of mutant strains (15). Conversely, virulence determinants are “the factors present in a microorganism that are responsible for the relative capacity of a parasite to cause damage in a host” (13), although perhaps a more useful, specific definition of a virulence determinant could be a gene enabling an organism to colonize the host successfully and which may then result in host pathology. An operational definition used for the identification of virulence determinants in experiments is a gene belonging to a pathogen whose inactivation or deletion leads to a loss of virulence of the pathogen and whose genetic reintroduction restores virulence (2). Consistent with this, virulence determinants are most straightforwardly defined as “requisite virulence factors” (11). The identification of virulence factors and virulence determinants for *Cryptosporidium* spp. is not straightforward and is even more complicated in particular settings such as host immune disorders. For simplicity and clarity in this review, we concur with and have adopted the terminology of Casadevall and Pirofski describing virulence factors as contributory but virulence determinants as requisite (11).

On a practical note, Edberg (16) stated, “For a microbe to generate disease, a number of sequential virulence factors must be active. While clearly the genes that code for virulence must be present in the microbe, disease generation is a phenotypic phenomenon.” An implication of this is that the presence of a virulence gene does not necessarily mean that it will be expressed or active, so the possession of a virulence factor gene does not necessarily equate with virulence *per se*. It is important to take this into account when considering appropriate methodologies for the detection and tracking of virulent strains. For instance, in many cases, genotype testing by PCR cannot definitively determine whether a strain is virulent but merely that it encodes a virulence determinant and thus is potentially virulent. In such cases, however, expression-based assays such as immunofluorescence assays (IFAs), enzyme-linked immunosorbent assays (ELISAs), or, indeed, mass spectrometry may be more appropriate alternatives, better able to discriminate a virulent phenotype (17).

Virulence factors are likely to be involved in adhesion, colonization, invasion, and host immune evasion. When characterized, most factors share one or more of the following properties: (i) they are externally exposed, either on the surface of the parasite or as secreted proteins; (ii) they are hypervariable between isolates; (iii) they are encoded telomerically or subtelomerically; (iv) they are multicopy or belong to gene families; and (v) they are glycosylated and/or lipoylated. Virulence factors have long been considered important microbial traits, and decades of research have been dedicated to the identification of such factors. Such efforts have been rewarded by the discovery of many and diverse microbial genes/molecules that mediate damage and disease in infected hosts. In bacteria, virulence factors are frequently located in pathogenicity islands and can be transferred via plasmids or lysogenic bacteriophages. Classical bacterial virulence factors include toxins, fimbriae, and flagella (18), while in single-celled eukaryotic pathogens, perhaps the best-characterized virulence factors are hypervariable surface proteins such as those that enable antigenic variation (19). Other surface virulence factors of protozoa also have well-established roles in adhesion (20), cell invasion (21), resistance to the host immune response (22), intracellular survival (23), and nutrient uptake (24), while secreted protozoan virulence

factors can vary considerably in character, from cytotoxic proteases (25), to signaling molecules capable of dysregulating homeostasis (26), to molecules involved in quorum sensing (27). In the postgenomic era, the discovery and validation of protozoan virulence factors in particular have been accelerated by the application of technological advances, including comparative genomics, transcriptomics, microarrays, and reverse genetics including gene replacement and small interfering RNA (siRNA).

When considering how each individual virulence factor contributes to an overall virulence phenotype, it is crucial to identify reliable measures of virulence (2, 8, 28). The ability of a microbe to cause disease in an animal model, which is central to Koch's postulate, has been the cornerstone of the measurement of virulence, but this relies on the availability of a susceptible experimental animal model (11). Some commonly used measures of virulence are mortality, morbidity, microbial burden, weight loss, condition, and the lifetime reproductive success of infected hosts versus uninfected hosts (2, 8, 28). Increasingly, additional measurements of virulence, such as different measures of host cell damage (both *in vitro* and *in vivo*) and the magnitude and type of inflammatory and immune responses elicited by the pathogen, are being introduced (11). The advent of such parameters provides researchers with useful laboratory-based and epidemiological assessments with which to consider the contribution of virulence factors to strain pathogenicity in cases where access to an animal model is not appropriate or feasible.

CRYPTOSPORIDIOSIS

Cryptosporidium was first described in the early 20th century; *Cryptosporidium muris* and *C. parvum* were the first species described (29, 30). The veterinary importance of *Cryptosporidium* spp. was highlighted by the associations of *C. meleagridis* with morbidity and mortality in turkeys in the 1950s (31) and of *C. parvum* with bovine diarrhea in the early 1970s (32). *C. parvum* is now regarded as an economically important cause of neonatal diarrhea in calves and lambs (33–39). Another species, *Cryptosporidium baileyi*, is recognized as an important cause of respiratory disease in poultry and game birds (40–43).

The first cases of human cryptosporidiosis were reported in 1976 in patients with severe watery diarrhea (44, 45). Debilitating disease, chronic infections, and high mortality rates identified in AIDS patients during the early 1980s led to the inclusion of cryptosporidiosis as an AIDS-defining illness. Immunocompromised HIV/AIDS patients with T cell counts of <50 are now regarded as being at the greatest risk (43, 46–51). The public health significance of cryptosporidiosis became apparent when *Cryptosporidium* was recognized as a common cause of acute diarrhea in immunocompetent individuals (47, 52, 53). *Cryptosporidium* spp. are now recognized as major waterborne parasites worldwide (54).

Cryptosporidiosis characteristically results in watery diarrhea that may sometimes be profuse and prolonged (33, 55). Diarrhea and abdominal pain are generally the symptoms which cause patients to seek medical attention, leading to a laboratory diagnosis of cryptosporidiosis. Other clinical features include nausea, vomiting, and low-grade fever. Occasionally, nonspecific symptoms such as myalgia, weakness, malaise, headache, and anorexia occur (33). The severity, persistence, and ultimate outcome of the infection are typically dependent on a variety of parasite characteristics and host factors. Host factors include both the immune status and

frequency of exposure of the infected individual; however, little is known regarding the pathogenic characteristics of *Cryptosporidium* spp. (55). The severity of a *Cryptosporidium* infection can vary from an asymptomatic shedding of oocysts to a severe and life-threatening disease. Immunocompetent individuals experience a transient self-limiting illness (up to 2 to 3 weeks). However, for immunocompromised patients, cryptosporidiosis can be a critical illness with persistent symptoms leading to dehydration and wasting (43, 56, 57) and was associated with significant mortality rates (58, 59). In addition, *Cryptosporidium* infection can cause atypical manifestations in immunocompromised patients, such as atypical gastrointestinal disease, biliary tract disease, respiratory tract disease, and pancreatitis (60). A review summarizing clinical features in immunocompetent and immunocompromised patients was recently published (61).

Although several *Cryptosporidium* species have been identified in humans, *C. hominis* and *C. parvum* account for more than 90% of human cases of cryptosporidiosis (62–64). Other species that have less commonly been associated with human disease globally include *C. meleagridis*, *C. cuniculus*, *C. felis*, and *C. canis*; however, this is setting dependent. Although the clinical presentations of *C. parvum* and *C. hominis* infections are very similar, several variants have been reported. Cama and colleagues reported differences in clinical manifestations among *Cryptosporidium* species and subtypes in HIV-infected persons and in children (65, 66), with *C. hominis* being associated mainly with diarrhea, nausea, vomiting, and malaise and *C. parvum*, *C. meleagridis*, *C. canis*, and *C. felis* being associated with diarrhea only. In addition, it was shown that *C. hominis* infection was associated with nonintestinal sequelae (joint pain, eye pain, recurrent headache, and fatigue), which were not reported for people infected with *C. parvum* (67).

Direct and several indirect routes of transmission of *Cryptosporidium* have been identified. Direct transmission occurs by the fecal-oral route from infected hosts, including animal-to-animal, animal-to-human (zoonotic), human-to-animal, and human-to-human (anthroponotic) transmissions (66, 68–70). Person-to-person spread is well described as secondary cases, especially in families and in outbreak settings (71), including institutions such as day care centers and hospitals (72). Epidemiological and microbiological evidence for the zoonotic transmission of cryptosporidiosis comes from outbreaks involving veterinary students and researchers who had contact with infected young calves and involving children visiting farms where the same strain type was identified in animals and people. Indirect transmission involves contact with *Cryptosporidium* fecally contaminated material, including water, food, and fomites such as clothes and footwear. Indirect transmission also occurs through environmental contamination, usually involving the release of feces, sewage, wastewater, or slurry, often as overflow following heavy-rain events (73–75). Another mode of transmission, via the inhalation of oocysts, was reported for immunocompromised patients and for children (76–78) and was supported by data from experimental intranasal infections of piglets (79). The symptoms associated with this route are respiratory (laryngotracheitis) and could be accompanied by mild diarrhea.

Most of the data on transmission pathways for *Cryptosporidium* come from reports of outbreaks, the majority of which are waterborne (54, 80–83). However, outbreaks represent only a small proportion of recorded cryptosporidiosis cases. The transmission routes for endemic diseases may or may not be the same as

TABLE 1 *Cryptosporidium* risk factors determined by case-control studies in developed nations, reported since the early 1990s^a

Reference	Location	No. of cases	No. of controls	Significant risk factor	Odds ratio (95% CI) ^b
85	Melbourne, Australia	201	795	Eating uncooked carrots	0.6 (0.4–0.9)
				Swimming in public pool	2.7 (1.9–3.8)
				Children aged <6 yr at home with diarrhea	7.4 (4.0–13.8)
				Persons aged >5 yr at home with diarrhea	1.8 (1.1–2.9)
				Animal contact in home	0.6 (0.4–0.8)
				Calf contact away from home	2.9 (1.5–5.7)
				Drinking unboiled water from river, lake, or dam	1.5 (0.8–2.7)
85	Adelaide, Australia	134	536	Eating uncooked carrots	0.6 (0.4–0.9)
				Swimming in public pool	1.2 (0.8–1.9)
				Children aged <6 yr at home with diarrhea	8.6 (4.8–15.6)
				Persons aged >5 yr at home with diarrhea	3.7 (2.2–6.2)
				Animal contact in home	0.6 (0.4–0.9)
				Calf contact away from home	5.1 (1.5–17.3)
				Drinking unboiled water from river, lake, or dam	3.1 (1.5–6.5)
86	USA	282	490	Contact with persons (>2 to 11 yr old) with diarrhea	3.0 (1.5–6.2)
				Contact with calves or cows	3.5 (1.8–6.8)
				International travel	7.7 (2.7–22.0)
				Freshwater swimming	1.9 (1.0–3.5)
				Eating raw vegetables	0.5 (0.3–0.7)
				Chronic medical condition	2.2 (1.2–4.0)
67	Wales and England	427	427	Travel outside UK	5.7 (2.9–11.2)
				Case contact	4.6 (2.4–8.7)
				Touching cattle	3.9 (1.4–10.0)
				Toileting child aged <5 yr	1.9 (1.1–3.2)
				No. of glasses unboiled water	1.1 (1.0–1.3)
				Eating ice cream	0.5 (0.3–0.7)
				Eating raw vegetables	0.5 (0.3–0.8)
				Eating tomatoes	0.6 (0.4–1.0)
87	Cumbria, England	152	466	Drinking cold unboiled tap water	1.4 (1.14–1.71)
				Visiting farms	2.02 (1.04–3.9)
88	Ontario, Canada	36	803	Swimming in a lake or a river	2.9 (1.2–7.4)
				Drinking municipal water	2.4 (1.04–5.7)
				Having a family member with diarrheal illness	2.9 (1.3–6.4)
89	Colorado	47	92	Swallowing untreated water from lake, river, or stream	8.0 (1.3–48.1)
				Exposure to recreational water	4.6 (1.4–14.6)
				Contact with child or diapers	3.8 (1.5–9.6)

^a Adapted from reference 69 with permission from Elsevier and updated with data from references 87, 88, and 89.

^b CI, confidence interval.

those in outbreak settings. This has been evaluated in case-control studies, the majority of which were reviewed by Yoder and Beach (84) and Hunter and Thompson (69). The key risk factors are the ingestion of contaminated drinking or recreational water, contact with infected persons or animals, travel to areas where the disease is endemic, and contact with children <6 years old (especially but not exclusively with diarrhea), as shown in Table 1. These investigations also showed a consistent negative association with the eating of raw vegetables. It has been suggested that repeated exposures to small numbers of *Cryptosporidium* oocysts on raw vegetables provide protective immunity. This hypothesis was supported by Frost and colleagues (90), who conducted a diary-based study for which participants were asked to record the occurrence of diarrheal or gastrointestinal symptoms. The authors reported that higher levels of anti-*Cryptosporidium* antibodies were associated with reduced rates of diarrhea. Those authors suggested that

the lower levels of illness in people with increased levels of antibodies may be due to enhanced immunity from repeated exposures to oocysts in drinking water. It is important to keep in mind that these cryptosporidiosis risk factors are relevant in industrialized nations and that the settings and challenges are very different in developing countries. In immunocompromised subjects, mainly those with HIV infection, several studies investigated the specific risk factors for this high-risk group and found that the drinking of tap water from an untreated supply, exposure to pets and animals, unsafe sexual activity, and the use of public toilets are associated with *Cryptosporidium* infection (91–93).

The epidemiology of *Cryptosporidium* has been investigated mainly at the genus level (85, 86, 94–98); however, as *C. hominis* and *C. parvum* have different host ranges and transmission cycles, it is likely that those epidemiological studies that looked at risk factors for both species combined will have emphasized risk fac-

tors common to both species and downplayed risk factors unique to one species or the other (69). To address this, a case-control study that discriminated *C. hominis* from *C. parvum* was performed with immunocompetent subjects, enabling the identification of species-specific risk factors (67). The major risk factor for *C. parvum* was the touching or handling of farm animals, while for *C. hominis*, the main risk factors were travel outside the United Kingdom and diaper changing, even of asymptomatic infants. Based on this observation, it was suggested that the main reservoir for *C. parvum* infection is indeed zoonotic, while for *C. hominis*, the most important reservoir may be asymptomatic carriage in young children. Subsequently, several epidemiological investigations using identification of isolates to the species level (99–101) and even subtyping (102–107) have been reported. One of the key findings was the description of a subtype of *C. parvum* (subtype IIc) that appears to be anthropogenic and not zoonotic (66, 108, 109). The identification of isolates to the species level has improved our understanding of the epidemiology of and risk factors for cryptosporidiosis and has been valuable in outbreak investigations (103). In recent years, the epidemiology of *Cryptosporidium* has been extensively reviewed (55, 60, 62, 69, 70, 84, 110–114).

CLINICAL DIAGNOSIS

The symptoms of cryptosporidiosis are not pathognomonic; laboratory verification is required to confirm the diagnosis. This is usually done by the detection (presence or absence) of oocysts in stool samples by a microscopic examination of smears stained with tinctorial stain (usually acid fast, such as modified Ziehl-Neelsen stain), fluorescent stain (such as auramine O), or immunofluorescent stain (115). Alternatively, oocyst antigen capture methods such as enzyme immune assays (EIAs) or immunochromatographic lateral flow (ICLF) assays may be used (115), and positive reactions must be confirmed by using a suitable confirmatory test (116). The detection of *Cryptosporidium* DNA by PCR is also used in some diagnostic laboratories (117), usually as part of a multiplex gastrointestinal pathogen panel or parasite panel, for example, with *Giardia duodenalis*, *Entamoeba histolytica*, and *Dientamoeba fragilis* (118). Commercial assays are becoming available, for which the target loci are not always revealed by the manufacturer, but for in-house *Cryptosporidium* detection assays, these include unspecified regions (119) and the small-subunit (SSU) rRNA gene (120).

The diagnostic sensitivities of routine tests have been compared, demonstrating the superior performance of immunofluorescence microscopy and PCR over comparable performances of EIAs and fluorescent stains, which in turn were better than acid-fast staining and ICLF assays (121). The specificity of microscopy depends largely on the skill of the microscopist to differentiate oocysts from other bodies in smears stained with nonspecific tinctorial and fluorescent reagents; immunofluorescent stains are more specific (115). Test selection will also depend on additional multiple attributes and the resources available to meet them, including technical expertise, turnaround time, performance in batch mode, hands-on time, consumables cost per test or batch, and specialist equipment (121).

For some patients, especially those who are severely immunocompromised, alternative samples may be more appropriate, and endogenous life cycle stages may be sought by the histological staining of gastrointestinal or other biopsy samples. PCR-based methods for the detection of *Cryptosporidium* DNA may also be

applied following tissue digestion and to sample types such as antral wash, bile, and bronchoalveolar lavage fluid specimens, as indicated by the patient's symptoms (122).

PCR detection from stool samples depends on effective DNA extraction, which is hampered by the robust oocysts, which require additional disruption steps, such as chemical treatment, bead beating, freeze-thaw cycles, or boiling, to access the sporozoite DNA. PCRs, where applied in routine diagnostic settings, are part of multiplex gastrointestinal parasite panels and can identify the *Cryptosporidium* genus but not the species. Species identification by PCR-restriction fragment length polymorphism (RFLP), PCR sequencing, or real-time PCR assays is usually performed as a reference test in specialist laboratories and is applied more comprehensively in some countries than in others (114).

There is no standardized subtyping method, although sequence analysis of the *gp60* gene has been applied to further characterize *C. parvum* and *C. hominis*; single-locus typing will underestimate diversity, as it ignores the potential for genetic recombination within *Cryptosporidium* sp. populations during reproduction (123), and standardized, multilocus subtyping schemes need to be validated to support epidemiological investigations (124).

CRYPTOSPORIDIUM

Cryptosporidium is an apicomplexan parasite of public health and veterinary importance. This phylum contains other medically important parasites such as *Plasmodium* and *Toxoplasma*. Most apicomplexan parasites are characterized by the presence of an organelle called an apicoplast. In addition, the invasive forms have an apical complex, comprising polar rings, rhoptries, micronemes, and conoid and subpellicular microtubules, which is involved in host-parasite interactions. Interestingly, *Cryptosporidium* spp. seem to have lost functional apicoplasts and mitochondria, as shown by genome sequence analyses.

The *Cryptosporidium* life cycle can be divided into six major developmental phases (33): excystation (the release of infective sporozoites), merogony (asexual multiplication within host cells), gametogony (the formation of micro- and macrogametes), fertilization (the union of micro- and macrogametes), oocyst wall formation (to produce the environmentally resistant stage responsible for the transmission of the infection from one host to another), and sporogony (the formation of infective sporozoites). *Cryptosporidium* species have a monoxenous life cycle completed within the gastrointestinal tract of a single host. During the whole cycle, the different forms are confined to the apical surfaces of the host cells. The infective forms attach to the apical surfaces by a poorly understood process and become internalized within an intracellular but extracytoplasmic compartment separated from the cytoplasm by an electron-dense layer that appears to be predominantly of host origin (125). The parasite is located within a closed compartment of the host cell plasmalemma, described as the parasitophorous vacuole (126), where it is protected from the hostile gut environment and is supplied with energy and nutrients by the host cell through a feeder organelle, which is unique among apicomplexan parasites (79). It has also been reported that *C. parvum* may have extracellular gregarine-like life stages (127), although no subsequent studies have so far confirmed this.

The established life cycle begins with the ingestion of the sporulated oocysts by the susceptible host; the oocysts undergo excystation and release four infective sporozoites (Fig. 1). The excysta-

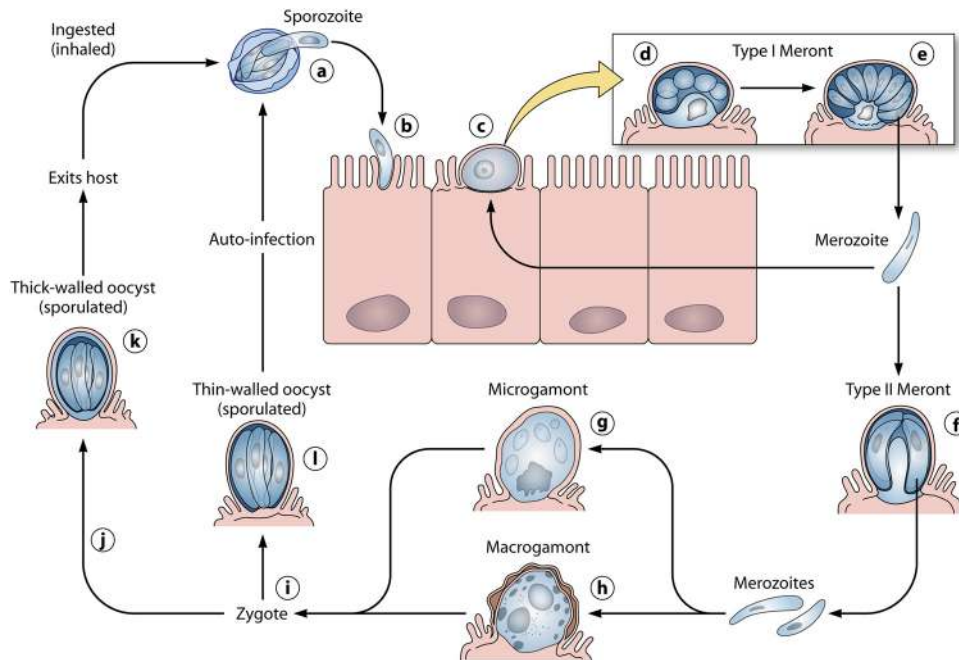


FIG 1 Schematic representation of the *Cryptosporidium parvum* life cycle. After excysting from oocysts in the lumen of the intestine (a), sporozoites (b) penetrate host cells and develop into trophozoites (c) within parasitophorous vacuoles confined to the microvillous region of the mucosal epithelium. Trophozoites undergo asexual division (merogony) (d and e) to form merozoites. After being released from type I meronts, the invasive merozoites enter adjacent host cells to form additional type I meronts or to form type II meronts (f). Type II meronts do not recycle but enter host cells to form the sexual stages, microgamonts (g) and macrogamonts (h). Most of the zygotes (i) formed after the fertilization of the microgamont by the microgametes (released from the microgamont) develop into environmentally resistant, thick-walled oocysts (j) that undergo sporogony to form sporulated oocysts (k) containing four sporozoites. Sporulated oocysts released in feces are the environmentally resistant life cycle forms that transmit the infection from one host to another. A smaller percentage of zygotes (approximately 20%) do not form a thick, two-layered oocyst wall; they have only a unit membrane surrounding the four sporozoites. These thin-walled oocysts (l) represent autoinfective life cycle forms that can maintain the parasite in the host without repeated oral exposure to the thick-walled oocysts present in the environment. (Modified from reference 33 with permission.)

tion of the oocysts has been reported to be triggered upon ingestion by various factors, including reducing conditions, carbon dioxide, temperature, pancreatic enzymes, and bile salts (40, 43, 128–130). Excystation allows the emergence of the four infectious sporozoites through a suture in the oocyst wall (129). The released sporozoites glide over the intestinal cell, releasing material from the apical complex (13). Gliding motility allows the sporozoites to migrate across the surface of host cells and to actively invade them (131). This form of motility is conserved among different apicomplexan parasites. *C. parvum* sporozoites undergo circular and helical gliding movements, which are actin-myosin-tubulin dependent (131, 132). During gliding motility, sporozoites deposit trails of proteins, which are involved in the attachment to and invasion of host cells (133). *Cryptosporidium* sporozoites possess an apical complex composed of micronemes, a single rhoptry, and dense granules (134). These secretory organelles are also present in other apicomplexan parasites, such as *Toxoplasma*, *Plasmodium*, and *Eimeria*, and are involved in host cell attachment and invasion (13, 135). The pellicle mediates movement and attachment to the host cell membrane via molecules exposed on the surface that drive the movement of the sporozoite, while the rhoptries and micronemes enable the sporozoite to adhere to and invade the cell, inducing the cell membrane to enclose the parasite in the parasitophorous vacuole (135–138). Each sporozoite develops into a spherical trophozoite, which undergoes merogony and forms a type I meront containing eight merozoites (43). These merozoites are released and attach again to the

surface of an epithelial cell, where they undergo merogony once more and form either a further type I meront or a type II meront. A type II meront contains four merozoites. These merozoites, when released, attach again to the epithelial cell, but instead of developing into further meronts, they initiate gametogony. Individual merozoites produce either microgamonts or macrogamonts (139, 140). Each microgamont undertakes nuclear division and differentiates to form up to 16 microgametes, which, when released from the parasitophorous vacuole, locate and fertilize a unicellular macrogametocyte that has developed from a macrogamont. The product of fertilization, the zygote, undergoes two asexual cycles of sporogony to produce an oocyst with either a thick wall or a thin wall, containing four sporozoites (141). The thick-walled oocysts are released into the lumen of the intestine, are excreted from the host in the feces, and are immediately infective, allowing the spread of infection to other susceptible hosts (140). In addition, unlike other parasites belonging to the same subclass (*Coccidia*), *Cryptosporidium* is able to autoinfect the same host. Autoinfection occurs through the thin-walled oocysts, which excyst once they are separated from the epithelium, and the cycle starts again (141, 142). Autoinfection and the recycling of type I meronts provide an explanation for persistent chronic infection (143–145).

GENOMICS OF *CRYPTOSPORIDIUM* SPECIES

Several studies tried to reveal the characteristics of the *Cryptosporidium* genome prior to the sequencing era. Karyotypic analyses

suggested that *Cryptosporidium* contains eight chromosomes, ranging in size from 0.945 to 2.2 Mb, giving a total haploid genome size of approximately 10 Mb (146, 147). In addition, *C. parvum* was shown to have two small extrachromosomal cytoplasmic virus-like double-stranded RNAs (1,786 and 1,374 nucleotides, respectively) (148). The RNAs have a single open reading frame each, which encode a putative RNA-dependent RNA polymerase and a protein with limited homology to mammalian protein kinases, respectively (149). Le Blancq and colleagues (150) investigated the *C. parvum* rRNA gene organization and reported that the small and large rRNA subunits are 1.7 and 3.6 kb, respectively, plus a 151-bp putative 5.8S rRNA. It was also demonstrated that *Cryptosporidium* does retain some mitochondrial biosynthesis genes (151) but that, unlike other apicomplexans, it lacks an apicoplast (152).

As a pathogen of public health relevance, *Cryptosporidium* was included in early genome-sequencing projects. Two reference strains served as genome representatives: *C. parvum* Iowa and *C. hominis* TU502. The genome sequences showed similar genome sizes of 9.11 and 9.16 Mb, respectively, with 3 to 5% sequence divergence (153, 154). In addition, the genome sequencing and assembly of *C. muris* strain RN66, a less relevant *Cryptosporidium* species from a public health perspective, are essentially complete (155). Genome analysis revealed extremely streamlined metabolic pathways and a lack of many cellular structures and metabolic pathways found in other apicomplexans (133). Energy metabolism is largely from glycolysis, and both aerobic and anaerobic metabolisms are available, thus conferring environmental flexibility (125). Limited biosynthetic capabilities and minimal metabolism have been reported, suggesting a large dependence on nutrient acquisition from the host (156). An extensive array of transporters was discovered, which enable the import of essential nutrients from the host (154, 157). Genome sequences showed that *Cryptosporidium* species have genes associated with apical complex organelles but suggested that they lack an apicoplast and possess only a degenerate mitochondrion which has lost its genome (153, 154). The existence of a relict mitochondrion was subsequently confirmed by ultrastructural studies (158). A comprehensive genome database, CryptoDB (<http://CryptoDB.org/>), serves as a public interface for *Cryptosporidium* genome sequences (159). This website offers access to sophisticated tools which enable the identification of genes based on text, sequence similarity, and motif queries (160).

The sequencing of *Cryptosporidium* genomes has revealed a vast amount of information, contributing to a better knowledge of microbial biology, pathogenicity, evolution, and virulence. The quest for the molecular basis of virulence has exploited these genomic data to search for genes that may ultimately unravel the regulation of virulence, host range, and transmissibility at the genetic level (11). Several comparative genomics studies have been performed since the completion of genome sequences from apicomplexan parasites of medical and veterinary importance. Templeton and colleagues (161) showed that *Cryptosporidium* spp. and *Plasmodium* spp. share over 150 ancestral "apicomplexan" proteins, involved mainly in interactions with eukaryotic host cells and the biogenesis of the apical complex. Gordon and Sibley (162) used genome sequences of *Toxoplasma gondii*, *Plasmodium* spp., *Cryptosporidium* spp., and *Theileria* spp. to show the conservation of actin-like proteins among these parasites, which rely on actin-based motility for cell invasion, while comparative

genomics of *Plasmodium* spp., *Cryptosporidium* spp., and *Toxoplasma gondii* revealed that calcium-regulated proteins (plant-like pathways for calcium release channels and calcium-dependent kinases) were also conserved (163). In addition to conserved genes, comparative genomics can identify unique, novel, and uncharacterized virulence genes. Kuo et al. (164) compared the genome sequences of three apicomplexan parasites (*Plasmodium*, *Theileria*, and *Cryptosporidium*) and showed that as many as 45% of the *Cryptosporidium* genes could be considered genus specific. Although the predicted gene counts were not identical, the papers reporting the genomes of *C. parvum* (3,952 genes) and *C. hominis* (3,994 genes) found no evidence for unique genes between the species, noting that what variation appeared to be present reflected primarily gaps in one genome or the other and speculating that the phenotypic differences likely arose from polymorphisms in coding regions and differences in gene regulation. The comparative genomics study by Kuo et al. (164), however, identified 334 putative *C. hominis*-specific genes and 178 putative *C. parvum*-specific genes by interrogations of the *Cryptosporidium* database into which the gene sequences had been placed. A similar *in silico* analysis that compared the genome sequences of *C. hominis* and *C. parvum*, aiming to uncover genetic loci responsible for host preference, showed 93 and 211 putative genes specific for *C. hominis* and *C. parvum*, respectively (63). However, when tested experimentally, the majority of the genes were found to be present in both species albeit with slight interspecies and intersubtype sequence variability. Nevertheless, PCR results showed experimental evidence for one *C. hominis*-specific and *C. parvum*-specific genes (165). A subsequent study by Widmer and colleagues which also aimed to investigate the genetic basis of *Cryptosporidium* host specificity used genome-wide comparisons of *C. parvum* zoonotic, *C. parvum* anthroponotic (IIC subtype), and *C. hominis* isolates (166). Those authors reported that for some genetic loci, there was actually more sequence similarity between *C. parvum* anthroponotic and *C. hominis* strains than there was between *C. parvum* anthroponotic and *C. parvum* zoonotic strains. A proteomic analysis of *C. parvum* proteins expressed during excystation showed an overexpression of three apicomplexan-specific and five *Cryptosporidium*-specific proteins (167). A recent *C. parvum* kinome analysis showed that several protein kinases of *Cryptosporidium* are distinct from those of other apicomplexans (*P. falciparum* and *T. gondii*) (168).

CRYPTOSPORIDIUM VIRULENCE FACTORS

Several studies have tried to determine the factors responsible for the initiation, establishment, and perpetuation of *Cryptosporidium* infection. *Cryptosporidium* does not normally cause a systemic infection or penetrate deep tissue; rather, the parasite establishes itself in a membrane-bound compartment on the apical surface of the intestinal epithelium (13). Nevertheless, it causes significant abnormalities in the absorptive and secretory functions of the gut. This damage could be the result of direct injury to the host epithelial cells or could be indirect through the effect of inflammatory cells and cytokines recruited to the site of infection (13).

Virulence factors are considered to be the processes and substances by which the parasite initiates and maintains disease in the host; these factors can affect the host at any time during the life cycle from the time when the parasite enters the body until it is killed or completes the cycle and exits the host (14). To date,

TABLE 2 Putative *Cryptosporidium* virulence factors identified by immunological and molecular methods^a

Virulence factor	Putative function(s)	Reference(s)
Serine protease	Excystation	229
Aminopeptidase	Excystation	169
CSL	Adhesion	170
gp900	Adhesion	137
gp60/40/15	Adhesion	171–173
P23	Adhesion, locomotion	174, 175
P30	Adhesion	176
TRAP-C1	Adhesion, locomotion	177
Cp47	Adhesion	178
CPS-500	Adhesion, locomotion	179, 180
Cp2	Invasion, membrane integrity	277
Cpa135	Invasion	181
Secretory phospholipase	Invasion, intracellular establishment	182
Hemolysin H4	Membrane lysis	183
CpABC	Transport, nutrient transport	184
CpATPase2	Biomembrane heavy metal transporter	185
CpATPase3	Biomembrane ion or phospholipid transporter	186
HSP70	Stress protection	187
HSP90	Stress protection	231
CpPKS1	Unknown function	188
Cysteine protease	Immune/cytokine modulation	189
Acetyl cosynthetase	Fatty acid metabolism	190
CpSUB	Invasion	191
CpMIC1	Adhesion, locomotion	192
CpMuc	Invasion	193

^a Data were compiled from data obtained from references 13, 14, 135, 191, 192 and 193.

Cryptosporidium-specific virulence factors have not been characterized to the point of unequivocally establishing their roles in causing damage to the host or proving that their deletion or inactivation results in a decrease of disease severity (13). This is mainly because, unlike other apicomplexan parasites such as *Toxoplasma* and *Plasmodium*, it remains difficult to employ *in vitro* cultivation and reverse genetics techniques with this parasite, meaning that genes cannot be readily knocked out or knocked down in experiments designed to examine virulence by a straightforward loss or gain of pathogenicity (133).

Putative virulence factors for *Cryptosporidium* have been identified as genes involved in the initial interaction processes of *Cryptosporidium* oocysts and sporozoites with host epithelial cells, including excystation, gliding motility, attachment, invasion, parasitophorous vacuole formation, intracellular maintenance, and host cell damage (14, 133).

Adherence Factors

A critical initial step in establishing infection is parasite attachment to host cells. Two classes of proteins, namely, mucin-like glycoproteins and thrombospondin-related adhesive proteins, have been characterized and shown to mediate adhesion (133). The characteristics of these proteins are summarized in Table 2.

CSL (circumsporozoite-like glycoprotein), with a molecular mass of ~1,300 kDa, was described by Riggs and colleagues (170) and is associated with the apical complex of sporozoites and merozoites (194, 195). CSL is released as a soluble glycoprotein and contains a ligand that binds specifically to a receptor on the surface of human and bovine intestinal epithelial cells (194). The zoite

ligand was shown to be involved in attachment and invasion (196). Monoclonal antibodies to CSL elicited changes in sporozoites and merozoites, similarly to the malarial circumsporozoite precipitate (CSP) reaction, and caused the complete neutralization of sporozoite infectivity (170).

Glycoprotein 900 (gp900) is a large glycoprotein identified by the immunoprecipitation of sporozoite extracts with hyperimmune bovine colostrum (137). This large mucin-like glycoprotein is located in micronemes and at the surface of invasive merozoites and sporozoites. gp900 is deposited in trails during gliding motility and is known to mediate invasion (197, 198). The deduced amino acid sequence of gp900 has a signal peptide and a transmembrane domain (197). Specific antibodies to gp900 can competitively inhibit infection *in vitro* (197, 199). gp60 N- and C-terminal peptides include a hypothetical signal sequence and a glycosylphosphatidylinositol (GPI) anchor attachment site, which are highly conserved among all *Cryptosporidium* isolates, suggesting that both features are important (171). The rest of the gene has a high degree of polymorphism, which is far greater than any other *Cryptosporidium* protein-encoding locus (109, 171, 200). A typing technique based on the sequence analysis of this polymorphic sporozoite surface glycoprotein has been extensively used and allowed the classification of *Cryptosporidium* isolates into gp60 subtypes (108, 171, 201–210). Interestingly, some gp60 subtypes were associated with strain virulence (65). Cama and colleagues (65) reported differences in clinical manifestations between *Cryptosporidium* species and subtypes in HIV-infected persons and in children (65, 66). Similarly, a study in China showed that two subtypes were associated with extended outbreaks in hospitalized children (211).

A sporozoite and merozoite cell surface protein gp15/40/60 complex has been described (Cp gp40/15 in *C. parvum* and Chgp40/15 in *C. hominis*): Strong and colleagues (171) reported that gp15/40/60 mRNA is translated into a ~60-kDa glycoprotein precursor during the intracellular stages of the *C. parvum* life cycle. Independently, Cevallos and colleagues (172) cloned and sequenced the same gene from *C. parvum* genomic DNA and called it Cp gp40/15. Shortly after synthesis, the 60-kDa precursor is proteolytically processed to generate 15- and 45-kDa glycoproteins. gp40 is localized at the surface and the apical region of the parasite and is shed from the surface, while gp15 is on the surface of sporozoites and is shed in trails during gliding movement (135, 172). Proteins present in the trails of gliding zoites have been shown to play a role in parasite attachment, invasion, and motility (133, 172, 201, 212–215). In *C. parvum*, gp15 is attached to the membrane via a GPI anchor (216). Both gp40 and gp15 display O-linked α -N-acetylgalactosamine (α -GalNAc), which is thought to be involved in invasion and attachment, since lectins that recognize these determinants block sporozoite attachment (173, 217, 218). A monoclonal antibody against gp15 neutralized infectivity *in vitro* and passively protected against the disease *in vivo*. It was suggested that a protein complex of gp900/gp40/p30 is formed to mediate adhesion via lectin activity (176, 219).

P23 is a 23-kDa sporozoite surface protein that is antigenically conserved across geographically diverse isolates (174) and is deposited in trails during the initial stages of infection (175). P23 elicits antibody responses in animals and humans exposed to *C. parvum* (220–223). P23 has neutralization-sensitive epitopes, and monoclonal antibodies were found to significantly reduce infection in mice and protect calves against cryptosporidiosis (135).

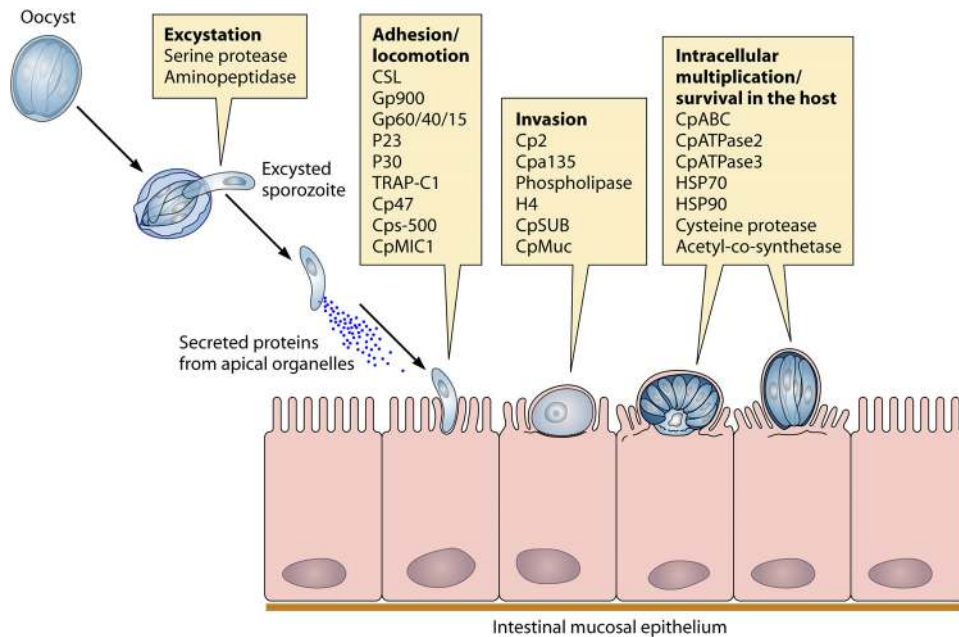


FIG 2 *Cryptosporidium* virulence factors described to date and their contribution to the parasite life cycle.

Of the micronemal proteins (MICs), TRAP-C1 (thrombospondin-related adhesive protein *Cryptosporidium* 1) is a 76-kDa protein localized on the apical pole of sporozoites (177). It showed sequence and structural homology to members of the thrombospondin family of adhesive proteins in other apicomplexan parasites (*Plasmodium* spp., *Toxoplasma gondii*, *Eimeria tenella*, and *Neospora* spp.) (135). TRAP and structurally related proteins are involved in parasite gliding motility and cell penetration (135). Putignani and colleagues (192) characterized “CpTSP8,” from a family of 12 *C. parvum* thrombospondin-related proteins (CpTSP2 to CpTSP12), which were identified by using bioinformatic tools. Those authors showed that CpTSP8 is located at the apical complex of sporozoites and merozoites and is translocated onto the parasite surface, as is typical of MICs. Therefore, CpTSP8 was renamed CpMIC1. MIC proteins have been shown to be essential for host cell attachment/invasion and gliding motility (224).

Gp900, gp40, gp15, Cpa135, Cp2, P23, and TRAP-C1 (Table 2) have or are predicted to have mucin-type O-glycosylation, suggesting that this type of posttranslational modification is common in proteins involved in attachment and invasion (133). Because mucin-like proteins were shown to be important for host-parasite interactions for *Cryptosporidium*, O’Connor and colleagues (193) undertook data mining of the *Cryptosporidium* genome databases to identify other mucin-like genes. They discovered a single locus of seven small mucin sequences (CpMuc1 to CpMuc7), which were expressed throughout the intracellular development stages. Specific antibodies inhibited infection *in vitro*, which is consistent with a role in host cell invasion.

The proteolytic processing of surface and apical complex proteins by parasite proteases has been shown to be required for the invasion of host cells and for egress from them. Further effort was focused on the identification of *Cryptosporidium* proteases. Wanyiri and colleagues (191) characterized a *C. parvum* subtili-

sin-like serine protease (CpSUB) and showed that this protein is likely to be responsible for the processing of gp40/15 (Table 2).

The identification of these molecules that mediate attachment to and invasion of epithelial intestinal cells (as shown in Fig. 2) is advancing our understanding of the cell biology of *Cryptosporidium* infection (219). These proteins have features in common with other apicomplexan proteins implicated in mediating host cell interactions and are expressed on the surface of the invasive *C. parvum* sporozoite and merozoite stages and shed in trails by gliding zoites (79, 171). The relative contribution of each individual molecule remains to be determined. It is likely that by using a large number of seemingly redundant adhesive molecules, the parasite can maximize the opportunity for cell attachment across a broad range of potential hosts. It is also possible that quantitative or qualitative differences in these glycoproteins may confer selectivity for host attachment (13).

Cellular Damage

Cell damage in enterocyte monolayers has been documented through the disruption of tight cell junctions, a loss of barrier function, the release of lactate dehydrogenase, and increased rates of cell death (225). The mechanisms that cause cellular damage during *Cryptosporidium* infection remain unknown; however, several molecules can cause direct tissue damage, such as phospholipases, proteases, and hemolysins (13).

Proteases have important functions in a parasite’s life cycle, such as mediating protein degradation, the invasion of host tissues, and the evasion of host immunity (226–228). Distinct protease activities have been identified for *Cryptosporidium* sporozoites: aminopeptidase, cysteine protease, and serine protease activities have been implicated in the excystation process (169, 189, 229). The identification of functional proteases in sporozoites during excystation and the prevention of infection in the presence

of protease inhibitors suggest that proteases are important in the initial stages of *Cryptosporidium* infection (13).

Hemolysin H4 has been identified by the screening of a *C. parvum* expression library on sheep blood agar (183). H4 has sequence similarity to the hemolysin of enterohemorrhagic *Escherichia coli* O157:H7 (183). The function of H4 is unknown, but its ability to disrupt cell membranes suggests a role in cellular invasion and/or the disruption of vacuolar membranes, which would allow merozoites to exit the parasitophorous vacuole and spread to adjacent cells (13). Another *Cryptosporidium* protein of interest is a *C. parvum* ATP-binding cassette (ABC) transporter gene (CpABC) localized in proximity to the electron-dense feeding organelle of the parasitophorous vacuole (184), which may well be associated with meeting key nutritional requirements. Interestingly, these genetic elements share structural similarities with bacterial genes, which are critical in producing secretory diarrhea (13).

Heat Shock Proteins

Heat shock proteins (HSPs) are a family of large conserved proteins. They are usually defined by their apparent molecular weights determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), with HSP90, HSP70, and HSP65 being common families (230). The level of synthesis of HSPs, especially HSP70, increases dramatically under stressful conditions (sudden shifts in temperature, decreased availability of nutrients, and immune attack). HSPs function as intracellular chaperones for other proteins; play an important role in protein-protein interactions; and facilitate the transport, folding, assembly, biosynthesis, and secretion of newly formed proteins (13).

For *Cryptosporidium*, two HSPs (HSP70 and HSP90) have been described (187, 231). Considerable polymorphism in the HSP70 gene has been identified and was used for genotyping purposes (232, 233). However, HSPs are under selective pressure, and their high degree of polymorphism might not reflect the genetic relationships among isolates or subtypes. For the closely related apicomplexan parasite *T. gondii*, it was demonstrated that quantitative and qualitative differences in HSP expression levels are related directly to parasite virulence (230, 234). High levels of expression of HSP70 were detected in virulent strains of *T. gondii* grown in mice, but little expression of HSP70 was observed in avirulent strains (235). The relationship between the level of HSP expression and *Cryptosporidium* virulence warrants further investigation.

Contingency Genes

Contingency genes, as opposed to conserved housekeeping genes, are highly variable (236). They are common in pathogenic microbes, including viral, bacterial, fungal, and protozoan pathogens (237, 238). Contingency genes are subject to spontaneous recombination rates higher than the background rate that applies to the other genes in the genome. These mutational events allow rapid switches in phenotype that are conducive to survival and proliferation in the host. In eukaryotic pathogens, these contingency genes are often associated with telomeres (239). Telomeres are prime sites for these quickly evolving genes that mediate host-parasite interactions. Examples of contingency virulence genes include the variant surface glycoprotein (VSG) in *Trypanosoma brucei* (239, 240), the var genes in *Plasmodium falciparum* (241), the trans-sialidases of *Trypanosoma cruzi* (242), the major surface gly-

coproteins (MSG) of *Pneumocystis carinii* and *Pneumocystis jirovecii* (243), and the subtelomeric variable secreted proteins (SVSPs) of *Theileria* sp. (244).

Comparative genomic analyses of *C. parvum* and *C. hominis* isolates showed that the most highly divergent regions are located near the chromosome ends (63, 166). The majority of such genes either were transporters or contained predicted signal peptides (166), a result which supports a role for telomeric contingency genes in dictating host specificity. Our comparative genomic analyses of *C. hominis* and *C. parvum* allowed the identification of a new family of telomerically encoded *Cryptosporidium* proteins: the *C. parvum*-specific protein (Cops-1) and the *C. hominis*-specific protein (Chos-1) (63). *Cops-1* and *Chos-1* appear to be distantly related and share similar characteristics, including having a telomeric location, encoding secreted glycoproteins of 50 kDa, and containing clear but distinct internal repeats. The proteins that they encode are predicted to be highly glycosylated, as shown in Fig. 3 (165). These characteristics suggested a possible role in host-parasite interactions, and we undertook a further characterization of the Cops-1 protein. Consistent with such a role, Cops-1 appears to be a secreted protein localized to the oocyst content and sporozoite surface of *C. parvum* but not *C. hominis* (165). Additionally, sera from *C. parvum*-infected patients recognized a 50-kDa protein in antigen preparations of *C. parvum* but not *C. hominis*, consistent with Cops-1 showing species-specific expression and being antigenic for patients (165).

Overview of *Cryptosporidium* Virulence Factors

The majority of the putative *Cryptosporidium* virulence factors detailed above, which mediate sporozoite attachment to and invasion of intestinal epithelial cells and the inactivation of which can prevent infection *in vitro* and *in vivo*, are common to all *Cryptosporidium* spp. These virulence factors were identified mostly in *C. parvum*. The number of *Cryptosporidium* species infecting humans is an ever-changing situation, and several new species/subtypes are added regularly. At the time of writing of this review, among 26 valid *Cryptosporidium* spp., at least 13 have been found in humans (with the most important for public health appearing to be *C. hominis*, *C. parvum*, *C. cuniculus*, *C. canis*, *C. felis*, and *C. meleagridis*). Of the >60 *Cryptosporidium* genotypes described, an increasing number are being identified in humans, including horse, monkey, skunk, and chipmunk genotypes, although it is uncertain if they are pathogenic. The virulence factors in human-infective *Cryptosporidium* species/genotypes other than *C. parvum* require investigation.

In addition to interspecies differences in virulence, there have been reports of intraspecies and interisolate virulence variability. Widmer and colleagues showed that *C. parvum* and *C. hominis* strains isolated from AIDS patients showed lower virulence than the GCH1 reference isolate in terms of the release of lactate dehydrogenase and the decrease in transmonolayer resistance (245). Okhuysen and colleagues used the 50% infective dose (ID₅₀) as a measure of virulence for three *C. parvum* isolates in healthy volunteers and showed that the ID₅₀ values were 9, 87, and 1,042 for strains TAMU, Iowa, and UCP, respectively (145). In addition, strain TAMU was the most virulent when the attack rate (clinical severity) and duration of diarrhea in humans were considered (86% and 94.5 h, respectively). Those authors associated this high level of virulence with fewer calf passages than the other two strains (with significantly higher passage rates).

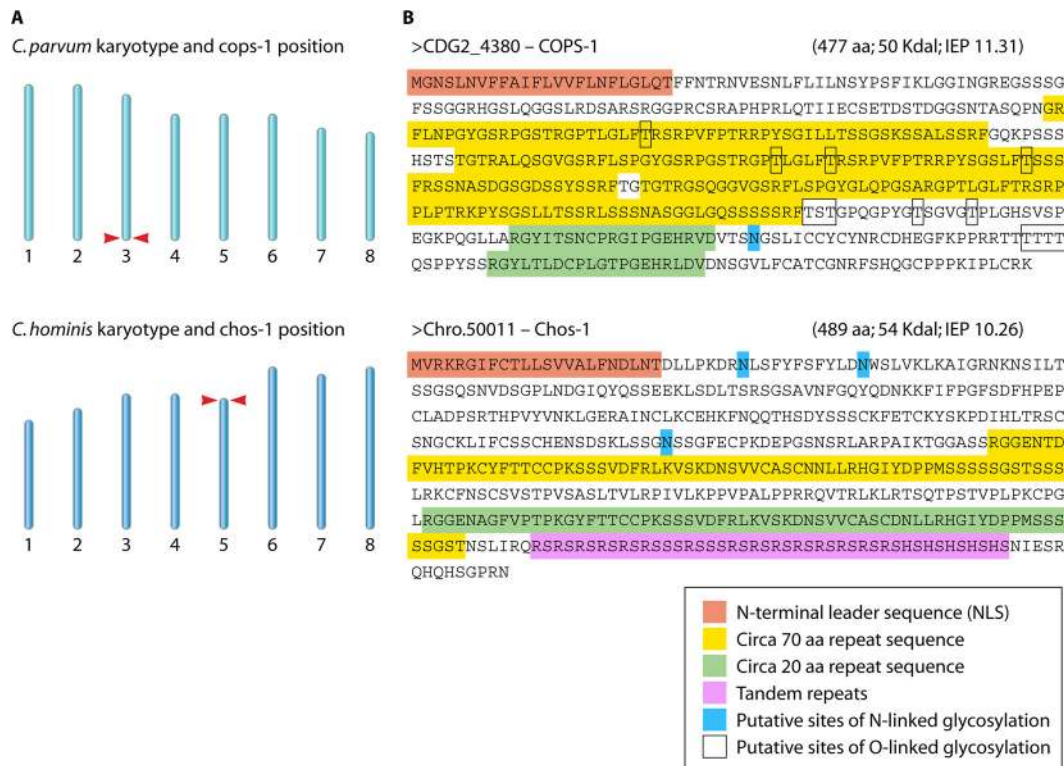


FIG 3 Characteristics of the new family of telomeric *Cryptosporidium* proteins, Cops-1 and Chos-1. (A) Genomic positions of the Cops-1 and Chos-1 genes. (B) Predicted Cops-1 and Chos-1 proteins are 50 kDa, secreted, and serine rich; contain internal repeats; and are highly glycosylated. aa, amino acids; IEP, isoelectric point. (Modified from reference 165, which was published under a Creative Commons license.)

Which genetic determinants dictate the virulence and host range of *Cryptosporidium* species and genotypes and by what specific mechanisms are not yet clear, and much work remains to be done. An improved picture of the composite biology of *Cryptosporidium* virulence factors is desirable and necessary in order to advance our understanding of the pathogenesis, host tropism, evolution, and epidemiology of *Cryptosporidium* species.

HOST FACTORS AND CRYPTOSPORIDIUM VIRULENCE

Host factors are critical determinants of the outcomes of host-pathogen interactions. It has been demonstrated that the severity of *Cryptosporidium* infection is highly dependent on host factors. The host immune capacity is the most important host factor affecting both the probability of an infection and the severity of the subsequent disease. This observation is most obvious when considering the impact of immune suppression on disease courses. Petry and colleagues (246) presented a comprehensive review of both innate and adaptive host immune responses to *C. parvum* infection. As the relevance and individual contribution of each of these responses remain unclear, the effect of any particular immunosuppressive disorder on the severity of *Cryptosporidium* infection remains difficult to predict (246). What is clear, however, is the link between an increased severity of disease and certain types of immune suppression. Indeed, many of the earliest-identified cases of human cryptosporidiosis were immunosuppressed individuals (33).

The severity of illness depends on the degree of the immunocompromised status (43, 57, 60, 61, 72). In immune-suppressed subjects, cryptosporidiosis is no longer self-limiting and can be

life-threatening (43, 57, 58). However, not all forms of immune suppression lead to an increased disease severity of cryptosporidiosis. The main risk seems to be immune-suppressive disorders that impact T cell function, the most obvious being HIV/AIDS (60). In AIDS patients, the most severe disease occurs in people with a CD4 cell count of less than 50. Flanigan and colleagues reported that in AIDS patients, self-limited cryptosporidiosis was seen in patients with higher CD4 counts (247). Further support for the role of T cell function was shown by improvements in the symptoms of cryptosporidiosis following treatment with highly active antiretroviral therapy (HAART) (248).

Of the primary immune deficiencies, those diseases that have been most clearly linked to an increased severity of cryptosporidiosis have included severe combined immunodeficiency syndrome (249), X-linked hyperimmunoglobulin M syndrome (250, 251), and CD4 lymphopenia (252). All three of these deficiencies are also characterized by impaired T cell function (253). In contrast, immune deficiencies affecting B cell function have not been so associated, although one report linked chronic intractable diarrhea and significant weight loss associated with cryptosporidiosis in an infant with a gamma interferon deficiency (254). There is also evidence that patients with malignant disease may also be more susceptible to infection, especially following bone marrow transplantation (60, 255, 256). However, the number of reports of *Cryptosporidium* in patients with malignancy is rather lower than for people living with HIV (257), probably reflecting a less common exposure in patients with malignancy than in patients with HIV infection. There have also been reports of cryptosporidiosis

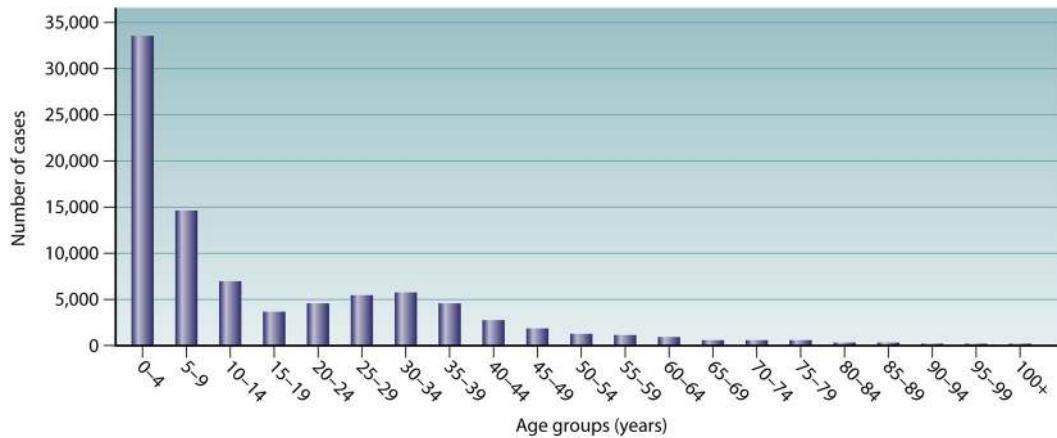


FIG 4 Total number of cryptosporidiosis cases detected in England and Wales by age group between 1989 and 2008, as reported by the Health Protection Agency (<http://www.hpa.org.uk/>).

in patients following solid-organ transplantation, although data were insufficient to make an adequate risk assessment (60, 255).

Acquired immunity would also appear to be important in preventing illness, as illustrated by reports of waterborne outbreaks of disease. During one such outbreak in Talent, OR, symptomatic cases of cryptosporidiosis occurred only in visitors to the town and not in residents, suggesting that residents had acquired immunity from previous exposures (258). For another outbreak, it was shown that swimming in a pool (a known risk factor) was negatively associated with disease in a drinking water-associated outbreak (259). One study showed that people with high anticryptosporidial antibody levels (IgG) were less likely to report diarrhea (260). What remains unclear is the duration of such acquired immunity. Evidence from adult human feeding experiments suggests that immunity may not last much longer than a year or so, and the protection acquired translates into less severe clinical symptoms (261). However, this is unlikely to be an all-or-nothing effect, as it appears that immunity merely shifts the dose-response curve to the right. In other words, even people who have acquired immunity can become ill if the ingested dose is sufficient; indeed, infection and diarrhea were reported in these subjects at a dose 20-fold higher than that for seronegative volunteers (260). There is also some evidence that such partial immunity may give rise to symptoms even though the oocysts remained undetected in stool samples. That same study showed that oocyst shedding occurred in only 53.8% of subjects with clinical cryptosporidiosis (260).

An interesting aspect of the issues around immunity is whether regular exposure to low doses of *Cryptosporidium* may actually have benefit. This notion was first raised independently by Craun and colleagues (262) and Hunter and Quigley (259), who reported that attack rates were significantly higher for outbreaks associated with groundwater than surface water consumption. They argued that people who use surface water sources were regularly exposed to small numbers of oocysts and thus did not experience many outbreaks, unless there was a major breakdown in treatment. The role of immunity in modulating virulence is also supported by a risk factor analysis which identified the eating of raw vegetable as a protective factor (Table 1). Using mathematical modeling, Swift and Hunter showed how an increased probability of exposure could lead to earlier infection but reduced risk later in life (263), a hypothesis supported by data from seroepidemiology studies

(90). Indeed, if, as recently hypothesized, repeated exposure serves to maintain an otherwise short-lived immunity, there would be an optimal daily probability of exposure that would minimize illness rates; however, any increased exposure would increase the risk to the youngest individuals, who are likely to be the most vulnerable (264). In a study modeling the risk of cryptosporidiosis associated with unreliable drinking water supplies in Africa, Hunter and colleagues argued that the main impact of increased exposure would be an earlier age of first infection (265).

The age of the host is a major factor in the epidemiology of infection, with children being most at risk for cryptosporidiosis, as shown by the cumulative data for cryptosporidiosis cases detected between 1989 and 2008 (Fig. 4). Illness in children is not necessarily more or less severe than that in older age groups, although they are more likely to report vomiting. In a case-control study, 70% of children (<17 years old) reported vomiting, compared to only 46% of adults (>16 years old) (P. R. Hunter, unpublished data). Infection maybe subclinical in the young, and asymptomatic carriage in children has been suggested to be an important reservoir for *C. hominis* infection (67). The high incidence of cryptosporidiosis in children probably reflects a lack of immunity due to few prior exposures and the immaturity of the gut mucosa (69, 72, 84).

Of particular relevance in developing countries is the issue of malnutrition in children. Cryptosporidiosis is common in children, and it is frequently associated with persistent diarrhea, malnutrition, stunted growth, and cognitive impairment (72). There is good evidence from animal studies that cryptosporidiosis exacerbates malnutrition and that cryptosporidiosis is more severe in malnourished mice (266). For humans, the evidence also supports this synergistic relationship (60, 267). However, the exact mechanisms behind the increased severity of illness in malnourished children are not yet fully clear. One likely explanation is that in a malnourished child, the damage to the gut mucosa with a new *Cryptosporidium* infection would lead to further impaired nutrient absorption and stunting that would not be such an issue for a well-nourished child (267).

Host behavioral factors also play an important role in the variation in the risk of cryptosporidiosis by increasing or decreasing the risk of exposure to the parasite. Several such factors are discussed above and include foreign travel, contact with livestock, swimming in a swimming pool, caring for a case of infection,

changing diapers, having multiple sexual partners, having pets, and using public toilets (51, 85, 86, 93, 268). These factors are likely to affect susceptibility to infection, but they are unlikely to affect infection courses or modify clinical manifestations.

HOST TROPISM AND VIRULENCE COEVOLUTION

The consideration of genetic determinants of host tropism as a virulence factor is legitimate, as these genes would enable the parasite to adhere to and invade epithelial cells from different host species. Indeed, the presence of closely related species that infect one host species and not another is a certain indicator of the presence of virulence genes, and as discussed above, there are clear differences between host tropisms among *Cryptosporidium* spp. and genotypes (113). *C. parvum* is fairly promiscuous and can infect a range of different species, while *C. hominis* appears to infect only humans. Other *Cryptosporidium* spp. also have distinct host ranges that may or may not include humans. In addition, some *Cryptosporidium* species are considered opportunistic; indeed, unusual species were reported to infect subjects of older ages or with immune disorders (269).

Nevertheless, the exact relationships between the host range and virulence of human-infective *Cryptosporidium* species is not clear. The Red Queen hypothesis postulates that species continually evolve but do not become better adapted (270, 271), and thus, interactions between hosts and parasites lead to constant natural selection for adaptation and counteradaptation (272). Antagonistic coevolution between hosts and parasites can involve rapid fluctuations of genotype frequencies and recombination in the host, which may be advantageous and can quickly produce disproportionately fit offspring (273). It is widely held that successful parasite species should evolve to become less virulent over time, with maladapted novel parasites being initially more harmful and then attenuating (274). Considering the host ranges of *C. hominis* and *C. parvum*, the latter might be anticipated to be more virulent, allowing it to infect a wide range of species, while the former is expected to be less virulent, as postulated by the theory of host-parasite coevolution and virulence attenuation. However, Hunter and colleagues (67) showed that *C. hominis* may be associated with an increased risk of postinfectious sequelae compared to *C. parvum*, while Cama and colleagues (66) showed that *C. hominis* was more virulent than *C. parvum* and other *Cryptosporidium* spp. in a developing-country setting, as measured by clinical symptoms and increased oocyst shedding. Based on similar observations, Woolhouse and colleagues (10) stated that host-parasite coevolution is in principle a powerful determinant of the biology and genetics of infection and disease, which has proven difficult to demonstrate rigorously in practice. A theory on the evolution of virulence states that pathogenicity can be maintained when it is a direct or indirect consequence of the parasite's exploitation of the host (274). Thus, in certain instances, such as when tissue damage is necessary to facilitate transmission, attenuation may be prevented during parasite adaptation to the host, and virulence may even be promoted (274, 275). This would seem to be the case for *Cryptosporidium*, where in order to maximize the potential for anthropogenic transmission, coevolution results in enhanced rather than diminished virulence.

The identification of genetic determinants of host tropism in human-infective *Cryptosporidium* species will improve our understanding of the evolution, virulence, and epidemiology of *Cryptosporidium* species and will afford improvements in our abil-

ity to identify and track virulent strains, particularly during outbreaks, improving opportunities for intervention. In addition, these virulence factors may well prove important as potential drug targets and vaccine candidates (11, 276). However, the identification and utilization of such determinants for these purposes have only just begun.

CONCLUSION

A major issue for virulence in *Cryptosporidium* is the difficulty in demonstrating clear phenotypic differences between many strains and species, especially in animals and cell culture. Nevertheless, by using molecular and immunological techniques, considerable advances in our knowledge of *Cryptosporidium* virulence and pathogenesis have been achieved recently. These advances were assisted by the publication of the genome sequences of three *Cryptosporidium* spp. The *trans*-expression of sporozoite proteins (Cpgrp40/15 and P23) in the related apicomplexan parasite *Toxoplasma gondii* (254, 255) has allowed for posttranslational modifications similar to those of the native protein and showed appropriate localization and glycosylation. The expression of *Cryptosporidium* proteins in this genetically tractable heterologous expression system may prove to be a major advance and would certainly assist in providing an improved understanding of the functional role of several *Cryptosporidium* proteins. In addition, with the establishment of a unique viral transfection system, advances in *Cryptosporidium* cell culture (successful long-term maintenance and propagation *in vitro*), and high-throughput next-generation sequencing of a wide variety of strains and species, our understanding of *Cryptosporidium* virulence is likely to continue to improve.

As summarized in this review, there are currently over 25 putative virulence factors, identified mainly by using immunological and molecular techniques. While no virulence determinant has been unequivocally identified to date and their composite biology remains unclear, it is likely that this will change in the near future as the transfer of new technology begins to enable direct testing. On the other hand, our knowledge of certain host factors that are associated with variations in both the severity and risk of infection has increased dramatically, although the mechanisms that lie behind these observations remain for the most part to be determined.

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