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Current Focus

Cryptosporidiosis: biology, pathogenesis and disease

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Abstract

Ninety-five years after discovery and after more than two decades of intense investigations, cryptosporidiosis, in many ways, remains enigmatic. *Cryptosporidium* infects all four classes of vertebrates and most likely all mammalian species. The speciation of the genus continues to be a challenge to taxonomists, compounded by many factors, including current technical difficulties and the apparent lack of host specificity by most, but not all, isolates and species.

Keywords: Cryptosporidiosis; *Cryptosporidium parvum*; Diarrhea; Enteric infection; Opportunistic infection

1. Historical perspective

Although *Cryptosporidium* was first described in the laboratory mouse by Tyzzer in 1907 [1], the medical and veterinary significance of this protozoan was not fully appreciated for another 70 years. The interest in *Cryptosporidium* escalated tremendously over the last two decades, as reflected in the number of publications, which increased from 80 in 1983 to 2850 currently listed in MEDLINE. The early history of *Cryptosporidium* is extensively documented in several review articles and book chapters published recently [2–4]. Taxonomically, *C. parvum* belongs to Phylum Apicomplexa (which possess an apical complex), Class Sporozoasida (which reproduce by asexual and sexual cycles, with oocyst formation), Subclass Coccidiasina (with a life cycle involving merogony, gametogeny and sporogeny), Order Eucoccidiida (in which schizogony occurs), Suborder Eimeriina (in which independent micro- and macrogamy develop), Family Cryptosporiidae (contain four naked sporozoites within oocysts—but with no sporocyst) [5]. Like other enteric coccidia of vertebrates, *Cryptosporidium* has a monoxenous life cycle that is primarily completed within the gastrointestinal tract of a single host. There are, however, many unique features that distinguish *Cryptosporidium* from other coccidia, of which the relative lack of host and organ specificity, resistance to antimicrobial agents, ability for autoinfection and the curious location it occupies within the host cell membrane are the most obvious [6].

Between 1980 and 1993, three broad entities of cryptosporidiosis became recognized [7]. The first was the revelation in 1980 that *Cryptosporidium* was, in fact, a common, yet serious, primary cause of outbreaks as well as sporadic cases of diarrhea in certain mammals [6]. From 1983 onwards, with the onset of the AIDS epidemic, *Cryptosporidium* emerged as a life-threatening disease in this subpopulation [8–11]. In 1993, it reached the public domain when it became widely recognized as the most serious, and difficult to control, cause of waterborne-related diarrhea [12]. The first glimpse of the seriousness of *Cryptosporidium* in mammals, mainly in calves, was provided in the late 1970s [13,14]. Until then, *Cryptosporidium* was mostly identified histologically in infected gut sections or in biopsy specimens [15,16] and was considered to be an opportunistic protozoan that caused a few or no symptoms.

2. Characteristics of the pathogen

Many aspects of the biology and the nature of *Cryptosporidium* interaction with the host cell remain unclear. The taxonomy of this parasite continues to be a serious challenge to biologists and molecular epidemiologists. While there appear to be clear differences among isolates of *Cryptosporidium* obtained from different sources, these differences at present are difficult to fully characterize or define phenotypically for the purpose of host specificity and

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speciation. The two major obstacles that hinder progress in this area are the inability to continuously propagate the parasite in vitro, and the inability to cryopreserve the parasite, as is the case with the majority of microorganisms. These technical problems are reflected in the absence of well-characterized reference strains of *Cryptosporidium* representing different vertebrate classes, species or genotypes. The few *C. parvum* isolates that are currently being used in laboratory investigations are maintained by passage through animals, mostly calves. There are only a handful of *C. parvum* isolates that are widely used and partially characterized genetically and phenotypically.

2.1. Species designation within the genus Cryptosporidium

In 1980, *Cryptosporidium* isolates obtained from calves, lambs and a human adult with severe diarrhea readily infected seven other species of animals [17]. The transmission of the human isolate, which induced acute diarrhea in lambs indistinguishable from that caused by other animal isolates, strongly indicated the potential zoonotic nature of *Cryptosporidium* [18]. Based on these early observations, the naming of *Cryptosporidium* species after their respective animal hosts [5] seemed questionable [17]. Subsequent studies extending over the last two decades, however, indicated that other species might exist [19]. *C. parvum*, however, the named mammalian species, remains the single most important species perpetuating the infection in mammals. The exact number of additional species is still unclear, but molecular methods are improving our understanding of the taxonomy of this genus. Of the original 21 different *Cryptosporidium* species listed, the majority became invalid as a consequence of the transmission experiments described above. At least six *Cryptosporidium* species are currently recognized, based largely on genotyping and a limited number of transmission experiments. These six species include two mammalian (*C. parvum* and *C. muris*) and two avian (*C. meleagridis* and *C. baileyi*) species, a species seen in reptiles (*C. serpentis*) and a species seen in fish (*C. nasorum*) [3]. Other less clearly defined species include those from guinea pigs (*C. wrairii*), cats (*C. felis*), dogs (*C. canis*) and marsupials (unnamed).

The current differentiation of isolates into valid species is based on their genetic profile and the species of the host from which they were originally isolated. There are serious limitations associated with speciation of *Cryptosporidium* based on the above criteria. For instance, since *C. meleagridis*, *C. canis* and *C. felis* were subsequently also observed in humans with cryptosporidiosis, these species would most likely have been named in relation to their human hosts rather than their current respective animal hosts. While these criteria, after some rigorous testing, are appropriate to other microorganisms, including other members of the Apicomplexa, they currently are premature with regard to *Cryptosporidium*. The reasons for this are several, including the ubiquitous nature of *Cryptosporidium* (at least *C. parvum*, the most extensively investigated species) and the technical barriers associated with propagation and maintenance. While there appear to be clear genetic and pathogenic differences among isolates of *Cryptosporidium* obtained from different classes of vertebrates, from different species therein or even from the same species of animals, these differences are inadequately characterized, particularly with respect to infectivity for various animals and the degree of virulence. The broader epidemiological and epizootiological implications of studies conducted on a few isolates, often using one animal species, carry major risks to public health. These can lead to a complacent view that humans are safe from exposure to *Cryptosporidium* that originates from non-mammalian vertebrates. Recent reports of human infections with *C. meleagridis* are a good example. On the other hand, the existence of diversity within *C. parvum* casts further doubts on speciation based on transmission experiments as well. *C. parvum* isolates, even when obtained from the same host (e.g. humans), display diversity in the range of mammalian species they infect. They have consequently been divided into genotype 1, found exclusively in humans and a few other primates, and genotype 2, found in most, if not all, mammals, including humans. Yet, in mixed infections (clinically and under laboratory conditions), each *C. parvum* type maintains a separate reproductive cycle, indicating a lack of genetic recombination between genotypes (Widmer, Akiyoshi and Tzipori, unpublished). Given such differences in genetic and infectivity profiles, they qualify to be considered as two distinct species.

The application of restriction fragment length polymorphism helped identify these two genotypes within *C. parvum*. This segregation was confirmed by a multilocus analysis based on polymorphisms (in microsatellites) located at five unlinked loci in the genome of *C. parvum*, applied to isolates from a variety of hosts and geographic origin [20,21].

We believe it is more than likely that speciation in the future will not necessarily follow along classes of vertebrates, but will rather be determined by tangible virulence attributes that can be linked to genetic markers. Demonstration of significantly greater sequence homologies within species than among species at multiple unlinked loci, in isolates obtained from a large and diverse range of host species and locations, could provide, in the future, a solid genetic basis for elucidating the taxonomy of the genus *Cryptosporidium*. Confirmatory evidence would require an experimental system to test whether putative species are reproductive entities.

2.2. Life cycle

The life cycle begins with the ingestion of oocysts by the host. Following excystation, four naked sporozoites are released in the gut, which then infect epithelial cells and

initiate asexual development. They become internalized and undergo two successive generations of merogony, releasing eight and four merozoites, respectively. The four merozoites released from the second merogony give rise to the sexual developmental stages, the micro- and macrogamonts. The release of microgametes, and their union with macrogametes, gives rise to the zygote, which, after two asexual divisions, forms the environmentally resistant oocyst containing four sporozoites, often while still within the parasitophorous membrane [2,7].

Fig. 1 provides a set of electron micrographs that illustrate the appearance of the various endogenous parasite stages, including developing and fully developed trophozoites (1 and 2), first- and second-generation merogony with eight and four merozoites within (3 and 4), the male microgametogeny with microgametes within (5), macrogamy (6), the fertilized zygote (7) and the walled-off oocyst still within the parasitophorous vacuole surrounded by the parasitophorous membrane (8).

The ability of the parasite to persist inside a single host is attributed to repeated first-generation merogony and the production of sporulated thin-walled oocysts, a characteristic quite distinct from other coccidia. The production and release of these oocysts within the same host are believed to be the key to autoinfection, a phenomenon observed by Tyzzer [22]. It is assumed that in the normal immunocompetent host, the infection remains localized in the gastrointestinal tract. Extraintestinal phases, however, should not be ruled out, as oocysts injected into the bloodstream of mice [23], or sporozoites into the peritoneal cavity (Tzipori, unpublished), lead to gut infection. The migration course of sporozoites from these sites into the gut is intriguing.

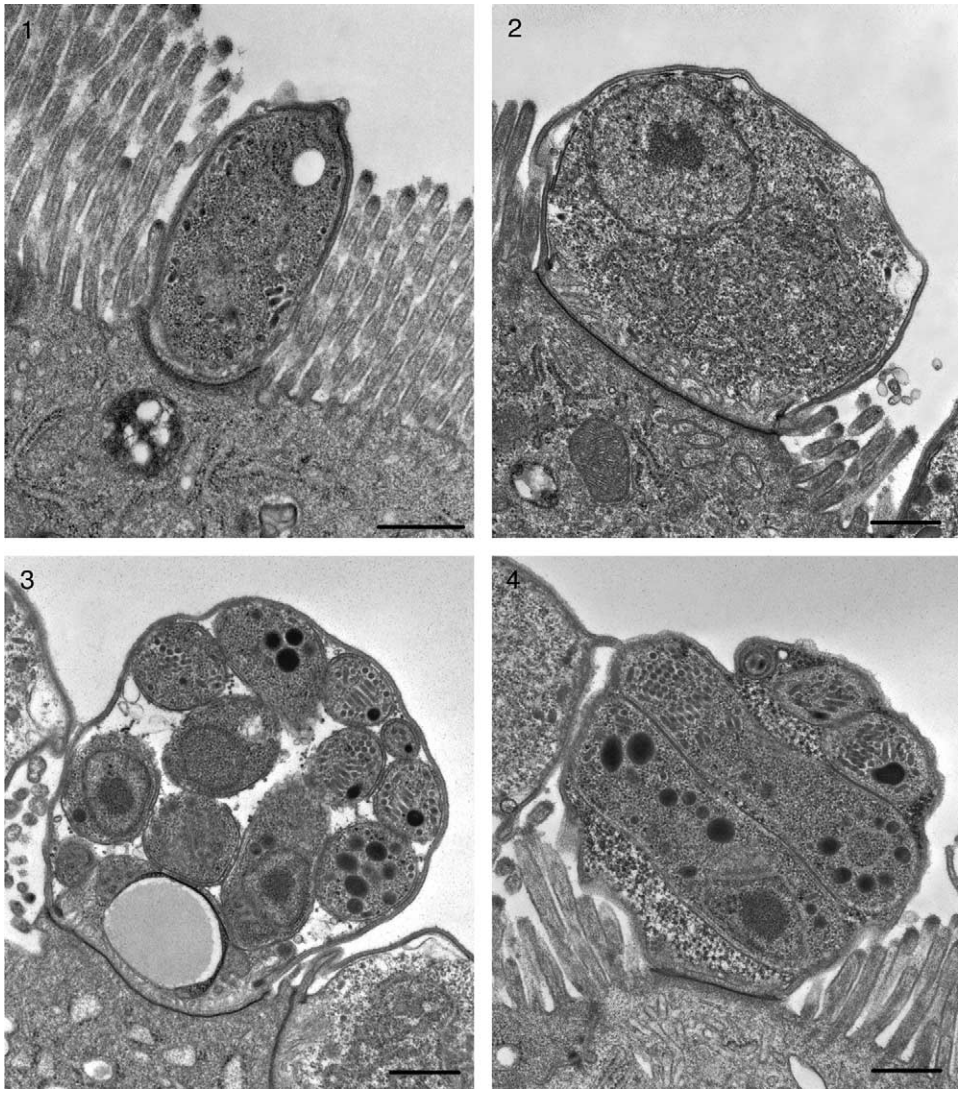

Fig. 1. Electron micrographs of gut sections from a piglet experimentally infected with *C. parvum* genotype 1. This is the first confirmed view of type 1 *C. parvum*. Bar = 500 nm. (Electron micrographs by Christine Pearson, Division of Infectious Diseases, Tufts University School of Veterinary Medicine). 1. Early development of a trophozoite after internalization. 2. Fully developed trophozoite before division into merozoites. 3. First generation schizogony with eight merozoites ready to be released into the gut lumen to infect new cells. 4. Second generation schizogony with four merozoites, which will give rise to either macrogamy or microgamy.

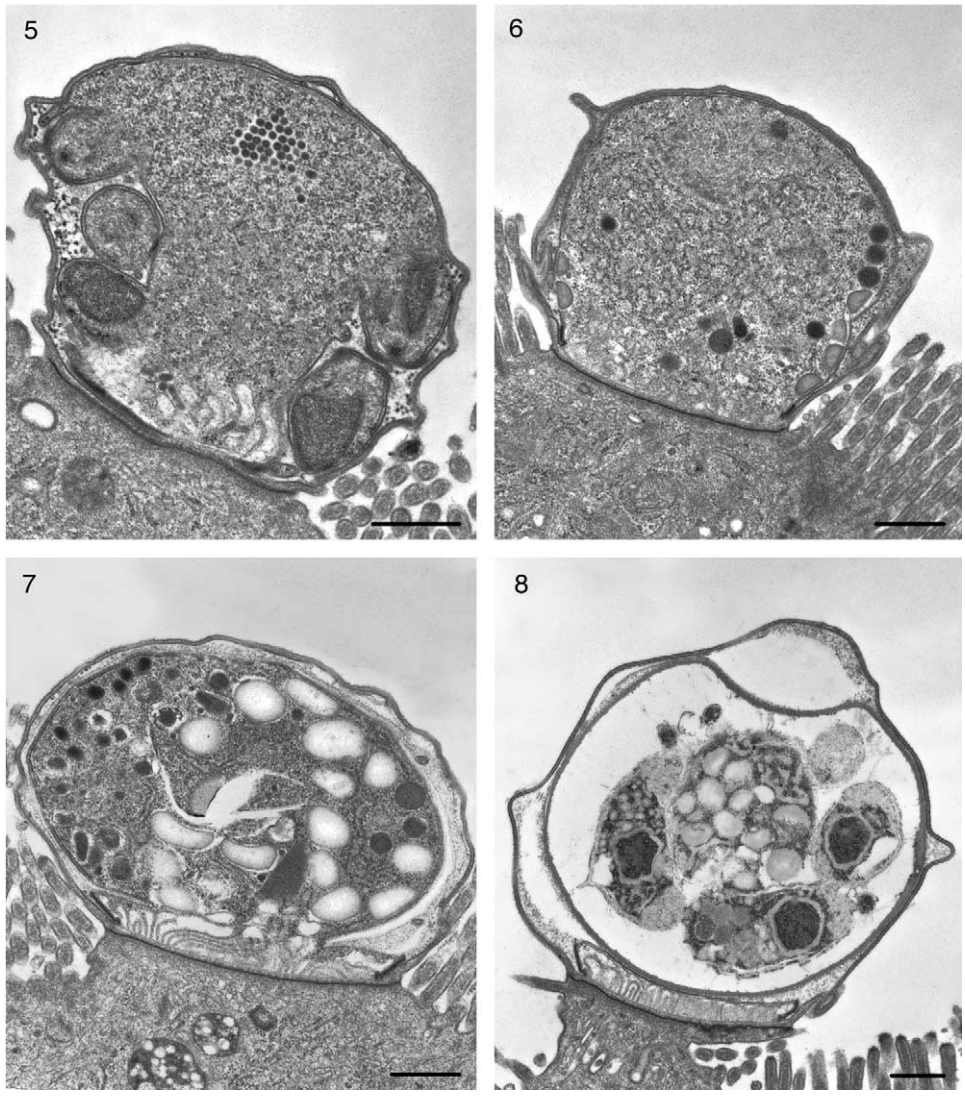

Fig. 1 (continued). 5. A dividing microgamy, which will give rise to some 16 microgametes, the male sexual stage. 6. A mature macrogamete, the female sexual stage, prior to fertilization. 7. A fertilized macrogamete, or zygote, walled off but still connected via the feeder organelle to the host cell cytoplasm. 8. A fully developed oocyst with four naked sporozoites within the thick and environmentally resistant oocyst wall. The oocyst is still within the host cell-derived parasitophorous membrane.

Extragastrointestinal phases have also been observed in *Eimeria tenella* and *E. maxima* [24,25].

2.3. Intracellular development

A detailed account of the invasion and internal development of *Cryptosporidium* as compared with other intracellular parasites, including other Apicomplexa, was published recently [7]. The degree of host tissue invasion by enteric pathogens ranges from non-invasive bacteria such as *Vibrio cholerae* and enterotoxigenic *Escherichia coli*, which cause no morphological changes to the microvillus border of enterocytes, to cellular invasion by pathogens such as enteric viruses, *Shigella*, *Salmonella*, *Coccidia* and *Eimeria* spp., to deep tissue invasion as seen in *Yersinia* spp., and *Salmonella typhi* and *S. paratyphi*. No organism other than *Cryptosporidium*, however, so extensively alters the cell membrane to create a niche for itself between the cell membrane and the cell cytoplasm. Neither the mechanisms of this process nor the implications in terms of accessibility to the parasite in this unusual location are understood. The location and the nature of this dual sequestration from the lumen of the gut and from the cell cytoplasm, one believes, hold the key to its enigmatic resistance to chemotherapy.

Cryptosporidium, like other coccidia, sequesters itself inside the host cell during development. It is protected from the host immune response and the hostile environment of the gut, while accessing the nutritional and energy reservoirs of the host cell. Again, like other coccidia, it lies within a parasitophorous vacuole bounded by a parasito phorous vacuolar membrane (PVM), which, in other coccidians, is the portal through which nutrients from the host cytoplasm enter the parasite. Unlike any other coccidian, however, *Cryptosporidium* has, in addition, a unique struc-

ture known as the feeder organelle membrane, which directly separates the cell and parasite cytoplasms. It is assumed that the PVM in *Cryptosporidium* provides only a protective function, while the feeder organelle membrane is the site for nutrient and energy uptake from the host cell. It is conceivable, however, that the PVM is also selectively permeable to certain molecules from the gut lumen. This is based on the fact that the PVM, originally derived from the host cell membrane, may retain some of its absorptive and other functional activities.

2.4. *Intracellular killing of* C. parvum

It is presumed that antimicrobial agents must first enter the host cell cytoplasm in order to effectively inactivate intracellular microorganisms. Other mechanisms involve the killing of the host cell containing the intracellular organism. The unique location of *C. parvum* below the cell membrane or the PVM, however, also gives it direct access to the extracellular domain, or the gut lumen. To investigate drug trafficking in *C. parvum*, we have used paromomycin, the only antimicrobial agent that partially, but consistently, inhibits parasite development in vitro and in vivo. These studies showed that paromomycin and geneticin, another aminoglycoside, mediate their in vitro inhibitory activity via a mechanism that does not require drug trafficking through the host cytoplasm. Apical, but not basolateral, exposure of infected cells to these drugs inhibited intracellular development, indicating an apical topological restriction of action [26]. These studies unexpectedly showed that the apical membranes overlying the parasite and the parasitophorous vacuole might, in fact, be the unsuspected major route of entry for paromomycin, and possibly of other drugs. These observations not only could explain the lack of efficacy of other agents active against intracellular parasites, but should also be considered when designing targeted, novel drug therapies against *C. parvum*.

3. Pathogenesis

The pathogenic mechanisms by which *Cryptosporidium* causes diarrhea, malabsorption and wasting are poorly understood. Whatever these mechanisms may be, the initial host–parasite interactions of attachment and invasion are critical primary events in pathogenesis. The ultrastructural characteristics of attachment and invasion and various factors influencing attachment have been described. However, little is known about specific parasite and host molecules involved in these processes [27]. Knowledge of such molecules is crucial for understanding the pathogenic mechanisms employed by this parasite.

The initial host–parasite interactions of attachment, invasion and parasitophorous vacuole formation are complex processes that involve multiple parasite ligands and host receptors. These interactions have been best studied in apicomplexans such as *Toxoplasma*, *Plasmodium* and *Eimeria*. Invasive “zoite” stages of apicomplexans possess specialized secretory organelles (rhoptries, micronemes and dense granules) collectively known as the apical complex. During initial host–parasite interactions, these organelles secrete and successively exocytose proteins, which facilitate attachment, invasion and parasitophorous vacuole formation. Many micronemal proteins have adhesive “modules” that are conserved among apicomplexan parasites, whereas others express unique domains [28]. Increasing recognition of *Cryptosporidium* as an emerging human pathogen has led to the identification of surface and/or apical complex proteins (such as CSL, GP900, p23/27, TRAP C1, GP15, CP 15, CP60/15, cp47, gp40/45 and gp15/Cp17) that have features in common with those of other apicomplexans and that are implicated in mediating these interactions. Many of these proteins have been reviewed previously [27,29]. Recently published findings on some of these proteins as well as those that have subsequently been discovered are described below.

The circumsporozoite-like antigen (CSL) is a highly glycosylated 1300-kDa glycoprotein, which was identified using a monoclonal antibody (mAb) to a repetitive carbohydrate epitope [30]. This mAb elicits a circumsporozoite-like reaction in which the antigen is translocated posteriorly along the sporozoite pellicle, resulting in loss of infectivity. This mAb also neutralized infection in vitro and in vivo in a mouse model of cryptosporidiosis. CSL is localized to the micronemes and dense granules of the apical complex and is also present on the surface of sporozoites and merozoites. The purified protein binds to host cells in a dose-dependent and saturable manner [31]. A recent study showed that CSL binds to an 85-kDa receptor on intestinal epithelial cells [32]. Taken together, these findings implicate CSL as one of the ligands mediating attachment and invasion; however, the molecular structure of this protein has not yet been described.

Like CSL, GP900 is a heavily glycosylated, high-molecular-weight glycoprotein that is synthesized in micronemes of the apical complex, secreted onto the surface of invasive zoite stages and shed in trails during gliding motility [33,34]. Analysis of the deduced amino acid sequence of the gene encoding GP900 indicates that it is a multidomain protein containing cysteine-rich and mucin-like domains, a transmembrane domain and a cytoplasmic tail [34]. GP900 displays extensive N- as well as O-linked glycosylation [33,35]. Purified native GP900 binds to intestinal epithelial cells and competitively inhibits *C. parvum* infection in vitro, as does the cysteine-rich domain of the recombinant protein as well as antibodies to this domain [27,35]. Taken together, these observations suggest that GP900 also mediates attachment and invasion. The relation ship, if any, between GP900 and CSL remains to be determined.

The thrombospondin-related adhesive protein of *Cryptosporidium*-1 (TRAP C1) [36] is a homolog of mi-

cronemal proteins, including TRAP, CTRP, CS, Etp100 and MIC-2 of the related apicomplexans *Plasmodium falciparum*, *E. tenella* and *Toxoplasma gondii*, respectively [28]. These proteins contain a conserved thrombospondin domain characterized by the presence of multiple thrombospondin-related motifs (TRMs) and have been convincingly shown to mediate attachment to host cells. The deduced amino acid sequence of TRAP C1 indicates the presence of an N-terminal signal sequence, a polyserine domain, a thrombospondin domain containing six TRMs, a transmembrane domain and a cytoplasmic tail [36]. Antibodies to recombinant TRAP C1 localize the protein to the apical region of sporozoites; however, there is no experimental evidence to suggest that TRAP C1 is involved in attachment or invasion.

More recently, we described gp40, another mucin-like O-glycosylated glycoprotein, which is localized to the surface and apical region of *C. parvum* invasive stages and is shed from the surface of the parasite [35,37]. gp40-specific antibodies neutralize infection in vitro, and native *C. parvum* gp40 binds specifically to host cells, suggesting that the protein is involved in adhesion and invasion. We have cloned and sequenced *Cpgp40/15*, the gene that encodes gp40 [38]. Analysis of the deduced amino acid sequence of this gene revealed an N-terminal signal peptide, a polyserine domain containing multiple predicted mucin-type O-glycosylation sites and a hydrophobic region in the C-terminal end consistent with that required for addition of a GPI-anchor. In addition to encoding gp40, *Cpgp40/15* encodes an immunodominant 15/17-kDa glycoprotein, localized to the surface of invasive stages, which is also implicated in host–parasite interactions [38–40]. gp40 and gp15 are derived by post-translational processing of a precursor glycoprotein that is encoded by *Cpgp40/15* and expressed in intracellular stages of the parasite [37,39]. gp40, the soluble N-terminal fragment, and gp15, the C-terminal portion of the gp40/15 precursor, appear to remain associated with each other following post-translational processing. Thus, gp15, which is anchored in the membrane via a GPI linkage [41], may serve as a "stalk" to link gp40 to the surface of the parasite.

A striking feature of the *Cpgp40/15* gene is the unprecedented degree of polymorphism, which is far greater than that of any other gene studied in *Cryptosporidium* to date [39,42]. In genotype 2 isolates, variation occurs mainly in the length of the N-terminal polyserine domain. However, in genotype 1 isolates, numerous single nucleotide and single amino acid polymorphisms define at least four allelic subgroups [39,42]. The finding of extensive polymorphism in the *Cpgp40/15* locus is consistent with its gene products being surface-associated virulence determinants that may be under host immune pressure, and indirectly supports a role for these glycoproteins in mediating infection. The *Cpgp40/15* gene is present in single copy and, in isolates of both genotypes, is expressed as multiple transcripts generated as a result of alternate polyadenylation [42]. Despite the extensive polymorphisms in the coding sequence of *Cpgp40/15*, the predicted signal sequence, GPI-anchor attachment site, proteolytic processing site, predicted O-glycosylation sites in the polyserine domain and the 3' UTR are conserved among isolates, suggesting an important role for these regions in structure and function [42].

Cp 47 is a membrane-associated protein, which binds to the surface of intestinal epithelial cells and is localized to the apical region of sporozoites. However, the gene encoding this protein has not been cloned [43].

Experimental and/or circumstantial evidence suggests that the proteins described above are involved in mediating attachment and invasion. However, progress in conclusively establishing the functional role of these proteins has been greatly hampered by the inability to propagate *C. parvum* in vitro and the lack of suitable transient or stable DNA transfection systems as have been developed for other apicomplexan parasites such as *Toxoplasma* [44].

4. Disease

Cryptosporidiosis is one of the commonest human enteric infections in developed and in developing countries. It is ubiquitous, zoonotic in nature, occurring in most, if not all, species of vertebrates, and can induce infection with as few as 10 oocysts or less in adult human volunteers [45]. It is not clear how hazardous oocysts from species of animals other than ruminants are to human health, but the risk is probably considerable, as there is evidence now that some avian (*C. meleagridis*) and other mammalian (*C. felis*, *C. canis*) species have been detected in humans with cryptosporidiosis [46–48]. Children acquire the infection mostly during or after weaning, and episodic disease occurs throughout life.

Exposure to *C. parvum* oocysts, either directly through contact with infected humans or animals, or indirectly by drinking or eating food washed with contaminated water, may lead to acute diarrhea. *C. parvum* causes an acute, self-limiting infection and diarrheal disease in immunocompetent people, in whom the onset may be rapid (3–7 d), depending on a combination of host (age, presence of maternal antibodies or previous exposure, and infectious dose) and parasite (origin and age of oocysts, and species/genotype) factors. Infection presumably begins in the small intestine, where the emerging sporozoites infect enterocytes, and after amplification, endogenous forms spread throughout the epithelial surfaces of both villi and crypts. The infection may spread throughout the gut, which includes the gastric mucosa and the small and the large intestines, or it may remain localized in segments of the small and/or large intestine. The extent of spread and the sites involved determine whether the infection is clinical or subclinical as well as the overall intensity of the disease. Generally, the more proximal in the small intestine the location is, the more severe and watery is the manifestation of diarrhea. Infections confined to the distal ileum and/or the

large bowel can often result in intermittent diarrhea or even be asymptomatic. Infections may often involve the pyloric region of the gastric mucosa. Parasite forms displace the microvillus border and eventually lead to the loss of the mature surface epithelium. The rapid loss of surface epithelium causes marked shortening and fusion of the villi and lengthening of the crypts due to acceleration of cell division to compensate for the loss of cells. The combined loss of microvillus border and villus height diminishes the absorptive intestinal surface and reduces uptake of fluids, electrolytes and nutrients from the gut lumen. The loss of the microvillus border in the proximal small intestine leads, in addition, to loss of membrane-bound digestive enzymes, whose role in children, in particular, is crucial, and contributes to marked maldigestion in addition to the malabsorption. Diarrhea lasting 7–10 d results in serious dehydration and loss of body weight. Specific antibodies are not considered to be a major factor in recovery from infection, although they may play a role in protection against reinfection. Although the immune factors that contribute to recovery from cryptosporidiosis in the immunocompetent host are not well understood, clearly the absence of optimal number of circulating or mucosal CD4 T lymphocytes, or interferon gamma (IFNγ), is critical [49]. Studies have shown that other cytokines and immune cells may play a significant role in recovery and protection against reinfection [27]. The role of immunity in disease and recovery are discussed in greater detail elsewhere in this issue.

4.1. Studies in human volunteers

The natural history, including infectivity and virulence, of *C. parvum* in healthy adult volunteers was initiated in 1993 at the University of Texas Health Science Center in Houston. Over a period of 8 years, the group has tested several *C. parvum* type 2 isolates in human volunteers [45,50–52]. These studies have contributed much information to risk assessment, as well as on the pathogenesis, epidemiology, immunology and management of human cryptosporidiosis.

Some 29 volunteers participated to determine the ID_{50} for the IOWA isolate of *C. parvum*, which was determined to be 132 oocysts [50]. A re-challenge study with the same volunteers conducted 1 year after the first challenge demonstrated that re-exposure to *C. parvum* is associated with a less intense infection and reduced severity of illness. The rate of infection after the second exposure was three of 19 (16%), as compared with the first challenge (12/19, 63%; $P < 0.005$). Although the rates of diarrhea were comparable after each of the two exposures, the clinical severity as determined by the mean number of unformed stools passed was less after re-exposure (12 vs. 5.5, $P < 0.05$) [51]. These studies reflected the relatively short duration of protection against *Cryptosporidium*, and that frequent exposure is required for full protection.

Further studies investigated the infectivity and pathogenicity of three additional type 2 *C. parvum* isolates. Table 1 suggests that infectivity (ID_{50}) and pathogenicity (duration of symptoms) do not correlate with one another.

4.2. Cryptosporidiosis in individuals with HIV/AIDS

Cryptosporidiosis is considered to be one of the most serious opportunistic infections that complicates AIDS. Individuals with CD4 T-cell counts of <150/ml who become exposed to *C. parvum* invariably develop persistent infection, with profound and life-threatening diarrhea [53]. Prolonged infections lasting several months or years in people with acquired [54] or congenital [55] immunodeficiencies often spread from the gut to the hepatobiliary and the pancreatic ducts, causing cholangiohepatitis, cholecystitis, choledochitis or pancreatitis. In chronically infected gut, the mucosal architecture undergoes gradual but profound disorganization, which includes disrupted epithelial surface, fibrosis, cellular infiltration and crypt abscessation [56]. Although the prevalence in people with AIDS is not high (5–15% in developed countries), the lack of effective treatment makes this infection the most troublesome among the opportunistic infections associated with AIDS.

4.3. Cryptosporidiosis in malnourished children

The association between chronic cryptosporidiosis, persistent diarrhea and malnutrition is not well established, although several reports indicate that children with malnutrition are more likely to develop persistent diarrhea. During a 15-month period of study conducted at the Mulago Hospital in Kampala, Uganda, some 63,200 children were seen in the Acute Care Unit, of whom 13,556 had diarrhea (incidence rate of 21.4%). A total of 2446 were enrolled in this cross-sectional study, meeting criteria of age (3–36 months), non-bloody diarrhea, and no other compli-

Table 1
Clinical outcomes of volunteers exposed to four distinct isolates of *C. parvum* type 2 isolates [45,52]

Isolate	Type	Animal source	ID_{50} in volunteers	Illness attack rate (%)	Duration of diarrhea (h)
UCP	2	Bovine	1042	59	81
Iowa	2	Bovine	87	52	64
TAMU	2	Equine	9	86*	94
Moredun	2	Cervine	300	69	122**

* $P < 0.05$, TAMU vs. UCP or IOWA.
** $P < 0.001$, Moredun vs. UCP or IOWA.

cating factors. Of these, 1779 (72.7%) had diarrhea, while 667 (27.3%) were recruited as controls (had no diarrhea). Among the 1779 children with diarrhea, 532 (29.9%) were classified as having persistent diarrhea (>14 d), and 1247 (70.1%) were classified as having acute diarrhea. Of the 1779 children with diarrhea, 72 (4.0%) were severely dehydrated, 365 (20.5%) had some dehydration, 1055 (59.3%) were still being breastfed, but only 10 (0.28%) were still exclusively breastfed (Tumwine and Tzipori, unpublished data).

There was a strong correlation between age distribution of diarrhea and the occurrence of *C. parvum*, between the ages of 3 and 36 months, after which the incidence of diarrhea and prevalence of *C. parvum* subsided considerably. Approximately 25% of the children at this age acquire the infection for the first time. The prevalence at this age group of other enteric protozoa, with the exception of *E. bieneusi*, such as *Giardia*, *Cyclospora* and *Entamoeba histolytica*, was minimal in our study.

Overall, 444 (25.0%) of the 1779 children with diarrhea had *C. parvum* compared to only 57 (8.5%) of the 667 children without diarrhea ($x^2 = 80.2$, $P = 0.0001$). PCR analysis performed on the 444 stools showed that 74% had type 1 (human), 19% had type 2 (zoonotic) and 6% had either a mixture of type 1 and 2, or different species of *Cryptosporidium*, including two *C. meleagridis*. Children with persistent diarrhea had a higher prevalence (31%) than children with acute diarrhea (22%), and the former was more common among children with stunted growth, or those who were underweight or wasted, than among nutritionally healthy children (Tumwine and Tzipori, unpublished data).

4.4. Mode of transmission and source of infection

C. parvum oocysts are released in large quantities from clinically infected humans and calves (>10^{10} during acute or chronic infections), and less from asymptomatically infected individuals and from other species of animals. Transmission is fecal–oral, either directly or indirectly via contaminated water or food washed or irrigated with fecally contaminated water. Human and dairy effluents are probably the most important sources of environment and surface water contamination.

C. parvum is one of the most serious and frequent causes of waterborne diarrhea. This is largely because of the small infectious dose required (<10 oocysts, depending on isolates). Oocysts are environmentally highly resistant to common disinfectants; they are discharged in large numbers by infected humans and animals, and via effluent disposal, find their way to surface water.

Until recently, only the mammalian species *C. parvum* was thought to be responsible for human disease, which has been detected in most, if not all, mammalian species [2–4]. Recent reports have, however, described human disease with *C. meleagridis*, a turkey respiratory *Cryptosporidium* described in 1955 [57]. *C. meleagridis* has been detected in patients with cryptosporidiosis [47] and in malnourished children with persistent diarrhea (Akiyoshi and Tzipori, unpublished data). Reports of other, less clearly defined isolates such as *C. felis* and *C. canis* have also been reported in sporadic cases of individuals with cryptosporidiosis [46–48]. It is not clear whether such unusual *Cryptosporidium* spp. only infect highly susceptible individuals with compromised immunity, such as people with AIDS and the severely malnourished, or healthy individuals as well. The *C. meleagridis* which was isolated from children in Uganda readily infected other mammals such as piglets and mice, as well as turkey and poults, indicating conclusively, for the first time, that some *Cryptosporidium* spp. are capable of crossing the vertebrate class barrier (Akiyoshi and Tzipori, unpublished data).

C. muris, the first mammalian species to be described [1], was observed in the stomachs of a few animals, including mice, rats, cats, dogs, cattle and camels. There has been only one unconfirmed case of *C. muris* in two healthy humans [58]. Oocysts of *C. muris* are larger than those of *C. parvum*, and the infection is asymptomatic and can be persistent.

The significance of *C. parvum* infection in domestic animals, newborn calves in particular, became evident in the early 1980s. The course of the infection and the disease it induces in a variety of small ruminants was reproduced experimentally, reported extensively and reviewed many times over [2–4].

C. parvum is probably present in every domestic cattle herd worldwide. Asymptomatic infections and prolonged oocyst excretion by adult cattle have become recognized as another major and continuous source of environmental contamination, and clearly the source from which newborn calves contract the infection at a very young age. *C. parvum* is also common in sheep, swine and goat herds, but prevalence is not as well documented [2,7].

Infections in dogs [59], cats and horses have been reported, and must be regarded as a potential source for human infection. However, *C. parvum* is not known to cause diarrhea in these animals [2,7], a vehicle normally responsible for massive production and environmental dissemination of oocysts. The prevalence of cryptosporidiosis in these species of animals is not extensively documented.

Wild animals, which are commonly infected with *Cryptosporidium*, also contribute to environmental contamination and disease transmission. Until we have methods to identify key virulence factors associated with infectivity and pathogenicity of isolates for humans from such sources, and until we are able to confidently speciate clinical isolates, *Cryptosporidium* from all sources, including birds and lower vertebrate animals, should be regarded as potentially hazardous to public health.

There is evidence that in addition to the oral–fecal route, transmission may also occur by inhalation of oocysts. Pulmonary cryptosporidiosis has been described in individuals with AIDS [60], and in a child with laryngotrache-

itis, which was confirmed by tracheal aspirates [61]. The frequency of laryngotracheal infection in immunologically competent humans is unknown. It is possible that the respiratory phase of the disease, when it occurs, induces either mild or no symptoms. In one report, diarrhea due to cryptosporidiosis correlated with a higher percentage of children with mild respiratory symptoms (42%) than in children with diarrhea due to other causes (13%). These authors concluded that transient respiratory tract infection may be common in healthy children, and may contribute to person-to-person transmission [62]. Since we are acutely aware in this laboratory of the risks involved in contracting the infection while working with *C. parvum*, we take unusual precautions to avoid accidental infections when handling infected animals and tissues. Despite these stringent procedures, we believe that some individuals, who became ill with *C. parvum* type 1, could have become infected by inhalation of aerosolized oocysts in the animal facility. We also have evidence from studies in piglets that infection of the trachea is common [63]. It is unclear how common respiratory tract infection with *C. parvum* is as a precursor to gut infections. We have been able to establish infection in the gastrointestinal tract of gnotobiotic piglets by intranasal spray of oocysts and by transmission from infected to uninfected animals housed in the same facility but with no direct contact other than the air. These preliminary observations do strongly suggest that infection with *C. parvum* type 1 can be acquired by inhalation of airborne oocysts.

5. Diagnosis

Detection depends on the presence of intact oocysts in feces. Because clinically affected individuals excrete them in abundance, the diagnosis of *C. parvum* is not difficult. The earliest method of staining of a fecal smear with modified acid fast (MAF) remains the quickest (~15 min) and the easiest to perform if the sample is reasonably fresh. Oocysts measure 3–5 μm, are round and stain bright red, surrounded by a refractile rim, and often, 2–4 sporozoite nuclei can be observed. Other methods used in clinical diagnostic laboratories include direct or indirect immunofluorescence microscopy and ELISA. None of these techniques allows discrimination regarding the species of origin of the oocysts, or whether they are infectious or not. Only genetic techniques, including PCR, used in most investigative laboratories, can help determine the possible source and risk to human health.

6. Therapy

Despite decades of research on hundreds of chemo- and immunotherapeutic agents either in vitro or in vivo in animal models and clinical trials, there is still no specific therapeutic or preventive modality approved for cryptosporidiosis. Non-specific supportive treatment, including rehydration and nutritional supplementation, remains a mainstay of management of the clinical manifestations of cryptosporidiosis. In AIDS patients, reduction in viral load and concomitant rise in CD4 counts achieved by antiretroviral therapy results in rapid clinical improvement in symptoms as well as a reduction in oocyst excretion [64].

The reasons for this remarkable and tenacious resistance of *Cryptosporidium* to various antimicrobial agents are not known. As discussed above, likely factors include the distinctive localization and unique structural features of the parasite. Thus, unlike other Apicomplexa and, indeed, other intracellular parasites, *Cryptosporidium* occupies a unique intracellular, yet extracytoplasmic, niche between the host cell membrane and the cytoplasm, sequestering it from the intestinal lumen on one side and the host cytoplasm on the other.

6.1. Newer chemotherapeutic agents and approaches

Exhaustive reviews of the numerous chemo- and immunotherapeutic agents evaluated for anticryptosporidial activity have been previously published [65–67]. The aminoglycoside paromomycin continues to be one of the few antimicrobial agents that remains consistently in clinical use. This is despite a recent prospective double-blind, placebo-controlled ACTG trial of paromomycin in 35 adults with AIDS and CD4 counts <150, which reported that this drug was no more effective than placebo [68]. A recent approach to therapy has been to use combination chemotherapy. A small open-label study of a combination of paromomycin and azithromycin for 4 weeks followed by paromomycin alone for 8 weeks in 11 patients with AIDS and CD4 counts <100 reported a significant and consistent reduction in symptoms and oocyst excretion [69].

One of the newer chemotherapeutic agents to be evaluated is nitazoxanide (NTZ). NTZ is a nitrothiazolylsalicylamide derivative with broad-spectrum parasiticidal activity against protozoa, nematodes, trematodes and cestodes [70,71]. Its reported efficacy against these parasites led to trials of the drug for cryptosporidiosis. An uncontrolled trial of NTZ in 12 patients with AIDS-associated cryptosporidiosis in Mali reported a >95% reduction in oocyst excretion in seven individuals. In four of these seven patients, eradication or decrease in oocyst excretion was associated with complete resolution of diarrhea [72]. Sub sequently, a double-blind, placebo-controlled crossover study of NTZ in 66 AIDS patients in Mexico reported parasitological cure (no oocysts detected in fecal samples) rates that were significantly superior to the placebo response in 65% of the patients [73]. Of these, 86% of the patients also reported resolution of diarrhea. More recently, a prospective randomized, placebo-controlled, double-blind trial of the drug was conducted in immunocompetent individuals (50 adults and 50 children) with diarrhea due to cryptospo-

ridiosis in Egypt [74]. In this study, of 49 patients randomized to receive 3 d of nitazoxanide, 80% showed clinical improvement in symptoms after 7 d and 67% had no oocysts detected in the stools, compared to 41% with clinical improvement and 22% with parasitological improvement in the placebo-treated group. Clearly, further clinical trials of this drug alone and in combination with other drugs, such as paromomycin or arithromycin, in immunocompetent as well as immunocompromised patients are warranted.

While the urgent need for an effective treatment against the life-threatening impact of cryptosporidiosis in people with HIV/AIDS has subsided with the introduction of antiretroviral therapy, this only applies to developed countries. Cryptosporidiosis remains a debilitating disease in Third World countries, particularly in infants and young children, in whom it is associated with chronic diarrhea and malnutrition. It is anticipated, however, that with the pending completion of the genome sequencing of *C. parvum* (see the article by Widmer et al. in this *Current Focus*), new molecular targets will be identified for a more rational drug design against cryptosporidiosis.

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