

Cryptosporidiosis in developing countries

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Abstract

Globally, *Cryptosporidium* infection continues to be a significant health problem where it is recognized as an important cause of diarrhoea in both immunocompromised and immunocompetent people. In developing countries persistent diarrhoea is the leading cause of death in children younger than five years of age, where it accounts for 30 to 50 percent of those deaths. Encouragingly an increasing number of investigations in developing countries employ molecular tools, significantly improving the quality of epidemiological information. This improved *Cryptosporidium* monitoring, with appropriate molecular methods, in surface water, livestock, wildlife and humans, will increase current knowledge of infection and transmission patterns, and ultimately help to control *Cryptosporidium* via improved risk assessments in the future.

Key Words: *Cryptosporidium*, waterborne, zoonotic, and developing countries

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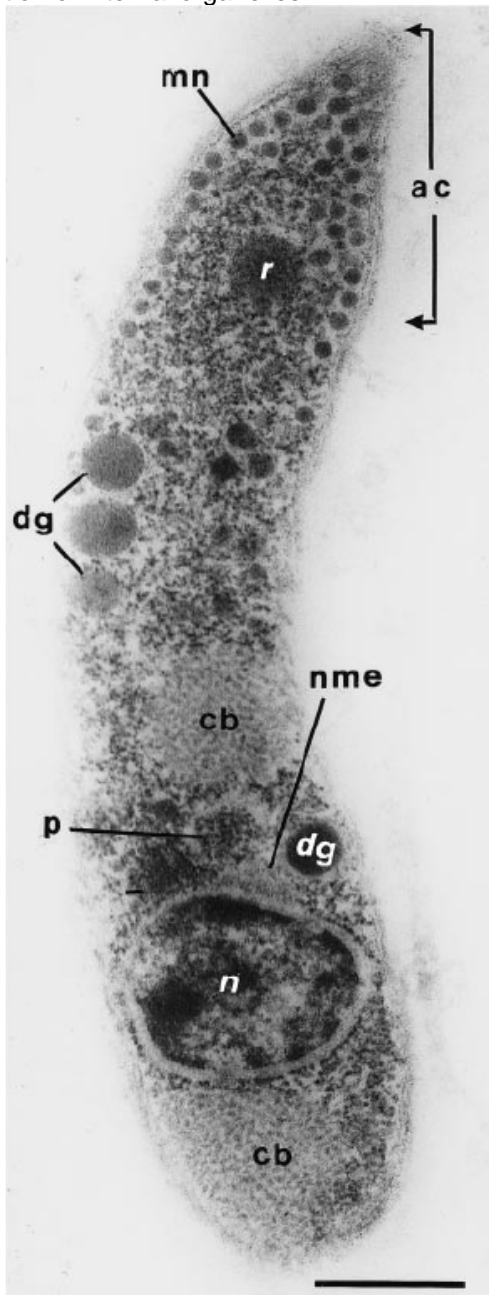
Introduction

The zoonotic intracellular protozoan parasite *Cryptosporidium* was discovered in mice by Tyzzer in 1907, but did not receive much interest from the scientific community for almost 75 years. However, *Cryptosporidium* research interest did intensify significantly in the 1980s due to increasing veterinary attention and the recognition of its impact on human health because of its association with the newly described acquired immunodeficiency syndrome (AIDS) [1]. Although research over the last two decades has dramatically increased our knowledge on *Cryptosporidium*, key questions about host-parasite interaction, cell-invasion, transmission, life cycle, and epidemiology still remain unclear [2]. This paucity of information is a reflection of our continuing inability to cultivate the organism to a significant degree in the laboratory and the

difficulty of obtaining and working with material derived from animal or human infection. *Cryptosporidium* is currently placed in the family Cryptosporiidae, within the phylum Apicomplexa [3]. Members of Cryptosporiidae have the common feature of four naked sporozoites, which are contained within a thick walled oocyst, and do not contain sporocysts [3,4]. The infective stage, the oocyst, is roughly 5 µm in diameter and contains four sporozoites each measuring 5 x 1 µm (Figures 1 and 2). *Cryptosporidium* is highly infectious and as low as 30 oocysts can cause infection in healthy volunteers [5]. *Cryptosporidium* oocysts are shed in large numbers in the feces of infected people or animals, are resistant to environmental stresses, and are able to resist standard disinfection, e.g. chlorination of drinking water, applied to drinking water [6]. There are currently 16 recognized

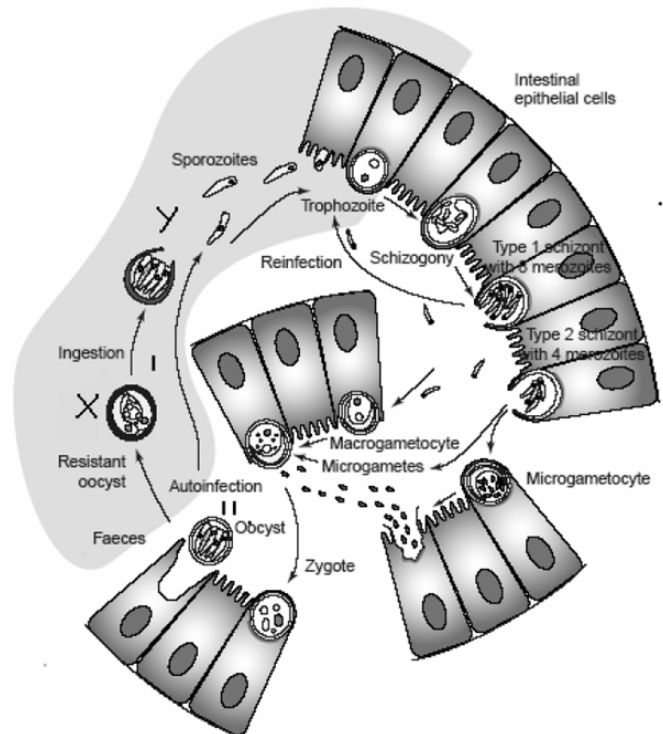
species of *Cryptosporidium*, which have been isolated from a large variety of hosts in all five groups of vertebrates, including humans (Table 1) [7].

Figure 1. The structure of *Cryptosporidium* [2,4,89,90]. Longitudinal section of a sporozoite showing the distribution of internal organelles.



The apical complex containing the micronemes (mn) and rhoptry (r) was at the tapering anterior of the cell (labelled ac) with the nucleus (n) and adjacent crystalloid bodies (cb) at the posterior, more rounded end. Dense granules (dg) occurred predominantly in the centre portion of the cell. The putative plastid-like organelle (p) and extended nuclear membrane region (nme) are also indicated. Scale bar 0 ± 0.5 µm.

Figure 2. Life-cycle stages of *Cryptosporidium* [2, 4, 89]. The stages of *Cryptosporidium* life-cycle, including both non-excysted (x) and (excysted) (y) stages.



Upon ingestion by the host, sporozoites are released and adhere directly to the intestinal epithelial cells of the host. Cell invasion by sporozoite is followed by intracellular development to trophozoite. Trophozoite undergo merogony to form meronts. Asexual replication occurs by re-infection of merozoites, released by type I schizont. Development of type II from type I meronts is the initial step of the asexual reproductive cycle. Merozoites are released from type II meronts and re-infect neighbouring cells where they develop into microgametocytes (male) or macrogametocytes (female). The macrogametocyte is fertilised by released microgametes and matures into a zygote, which undergoes further development into an oocyst. Two types of oocysts are released: (I) thick-walled oocysts, which are excreted in the faeces, or, (II) thin walled oocysts for endogenous re-infection (auto-infection).

Cryptosporidium infection continues to be a significant health problem in both developed and developing countries [10], where it is recognised as an important cause of diarrhoea in both immunocompromised and immunocompetent people [5,11]. Whilst other species including *C. felis* can also infect humans, in developed countries, the vast majority of human cases of cryptosporidiosis in the world are caused by *C. hominis* and *C. parvum* [12]. In developed countries various modes of transmission have been identified, among which are consuming contaminated water and food, through recreational water activities, close person-to-person contact,

e.g. hospital cross infections, and through zoonotic sources [13]. Large outbreaks due to the contamination of water supplies have been documented in recent years and in one particular outbreak, contamination of a water-treatment plant in Milwaukee was estimated to result in infections in 403,000 people [14]. Goldstein *et al.* (1996) later showed that this large outbreak was associated with municipal drinking water, despite state-of-the-art water treatment and water quality better than that required by current federal standards in the USA [15].

Table 1. Recorded species of *Cryptosporidium*, their size and major hosts [4, 8, 9].

<i>Cryptosporidium</i> species	Size (µm)	Host	Location
<i>C. andersoni</i>	5.5 x 7.4	Bovines	Abomasum
<i>C. baileyi</i>	4.6 x 6.2	Birds	Cloaca, bursa, respiratory tract
<i>C. canis</i>	5.0 x 4.7	Canids, human	Small intestine
<i>C. felis</i>	4.5 x 5.0	Felids, human	Small intestine
<i>C. galli</i>	8.0-8.5 x 6.2-6.4	Birds	Proventriculus
<i>C. hominis</i>	4.5 x 5.5	Human	Small intestine
<i>C. meleagridis</i>	4.5-5.0 x 4.6-5.2	Birds, human	Intestine
<i>C. molnari</i>	4.7 x 4.5	Fish	Stomach
<i>C. muris</i>	5.6 x 7.4	Rodents, human	Stomach
<i>C. parvum</i>	4.5 x 5.5	Ruminants, human	Intestine
<i>C. varanii</i>	4.2-5.2 x 4.4-5.6	Lizards, snake	Intestinal and cloacal mucosa
<i>C. serpentis</i>	4.8-5.6 x 5.6-6.6	Snakes, Lizards	Intestinal and cloacal mucosa
<i>C. suis</i>	5.1 x 4.4	Pigs, human	Stomach
<i>C. wrairi</i>	4.0-5.0 x 4.8-5.6	Guinea pigs	Small intestine
<i>C. bovis</i>	4.2-4.8 x 4.8-5.4	Ruminants	Small intestine
<i>C. scophthalmi</i>	3.0-4.7 x 3.7-5.0	Fish	Intestine

Traits of *Cryptosporidium* infection in developing countries

Persistent diarrhoea is the leading cause of death in children younger than five years of age in developing countries, where it accounts for 30 to 50 percent of childhood mortality [16]. Although many viruses, bacteria, and parasites can produce persistent diarrhoea, enteropathogenic *Escherichia coli*, enteroaggregative *E. coli*, *Giardia*, *Cryptosporidium*, and *Cyclospora* are several important agents [16]. *Giardia duodenalis* cysts,

microsporidia spores and *Cryptosporidium* oocysts have been detected in various ground water resources, but their role in community outbreaks and maintenance of the infection has not been fully characterized and better statistics exist for developed countries [6,17]. Most studies on prevalence have been carried out in developed countries, where the laboratory and other health infrastructure are more accessible than those in developing countries [17]. Whilst meaningful interpretation of population structures and occurrence-prevalence baselines can be performed, more useful data can be obtained by analysing a well-planned set of samples, taken from all possible sources, regularly over time, rather than focusing on outbreak investigations [18]. However, this is often more difficult to apply in developing countries. The lack of sample quality and relative inadequacy of laboratory diagnosis can affect accurate estimates of the prevalence of these infections in developing countries. Validated methods to determine the species, genotype and subtype that are present in heterologous mixtures should ideally be applied to environmental samples [18]. This is to enable the monitoring and characterization of infection sources, disease tracking and the establishment of causative links to both waterborne and foodborne outbreaks. With currently available tests, identifying a specific cause usually is difficult [16]. Whilst ideally more sensitive molecular tests should be used in studying the epidemiology of persistent diarrhoea in children [14], their application has been restricted by their relatively high cost [17].

Drinking water is a major source of microbial pathogens in developing regions, although poor sanitation and food sources are integral to enteric pathogen exposure [20]. *Cryptosporidium* is known to be a major agent of severe diarrhoea and opportunistic infection [12,21]. Emerging evidence shows that infection with *C. canis*, *C. felis*, and *C. meleagridis* show a higher prevalence in developing countries compared to developed countries [17]. Thus, inhabitants may also be more likely to be infected by *C. felis*, but the source and transmission routes for it are unclear [12,22]. *Cryptosporidium* is responsible for diarrhoeal diseases that may lead to nutritional deficiencies and significant morbidity and mortality, especially among children in developing countries

and patients who have immune defects, e.g. AIDS [17, 23]. Higher prevalence rates also tend to be observed more in rural compared to urban communities [17]. In Lima, Peru, the incidence of cryptosporidiosis peaks at 0.42 for 1-year-old children and declined to 0.06 episodes/child-year for 5- to 9-year-old children [24]. Cryptosporidiosis is more frequent during the warm season (December to May) than the cooler season (June to November). Cryptosporidiosis is more frequent in children from houses without a latrine or toilet [24]. In Brazil, the detection of zoonotic *C. parvum* in capybara (*Hydrochoerus hydrochaeris*), a semi-aquatic mammal that inhabits anthroponotic habitats, raised concerns that human water supplies may be contaminated with zoonotic *Cryptosporidium* oocysts from some wildlife [25].

Travellers' diarrhoea is also one of the most common health problems that afflict individuals from developed countries visiting less affluent regions of the world [26]. Reports of these infections in travellers and workers returning from developing countries can provide some indication of the extent of these problems [17]. Jelinek *et al.* (1997) examined 469 travellers returning to Germany with diarrhoea and detected 13 (2.8%) infected with *Cryptosporidium* [27]. Although travellers are aware of risk factors, they rarely exercise dietary precautions aimed at prevention [26].

Cryptosporidium research in developing countries

Over recent years, encouragingly an increasing number of more discriminatory studies are being performed in developing countries, increasing the amount of available data (Table 2). A survey conducted in the Southern Province of Zambia determined the prevalence of endoparasites and their association with diarrhoea using conventional and molecular analyses of stool and urine samples from school-age children (n = 93) [36]. Almost half of the stools (49.5%) were diarrhoeic. The overall prevalence of *Endolimax nana*, *Schistosoma haematobium*, *Blastocystis hominis*, *G. lamblia*, *Cryptosporidium* spp., *Encephalitozoon intestinalis*, and *Strongyloides stercoralis* were 64.3, 59.1, 53.8, 19.4, 8.6, 8.6, and 1.1%, respectively [36]. In a study in Malawi, DNA from 69 *Cryptosporidium*-positive human fecal samples were examined by multilocus

genetic analyses [34]. From 43, 27 and 28 of the samples, the SSU rRNA, 70 kDa heat shock protein (HSP70) and 60 kDa glycoprotein (GP60) genes, respectively, were successfully PCR-amplified [34]. Restriction analysis of the SSU PCR products showed that 41 of the 43 PCR-positive samples had *C. hominis* and two had *C. parvum*. Sequence analysis of the HSP70 and GP60 gene confirmed the species identification by SSU rRNA PCR-RFLP analysis, but also revealed high intraspecific variations. Altogether, six HSP70 subtypes and six GP60 subtypes (belonging to four subtype alleles) of *C. hominis* were found [34]. Thus, cryptosporidiosis in this study area was largely caused by anthroponotic transmission [34]. In another Malawian study, the incidence of cryptosporidiosis in children aged <five years with diarrhoea in an urban and rural hospital-based setting was examined [33]. Stools were collected over a 22-month period during both rainy and dry seasons. A range of microscopic methods were used to determine the presence of *Cryptosporidium* spp. oocysts. Species determination was carried out by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of oocyst-extracted DNA using 18S rRNA and COWP gene loci [33]. *Cryptosporidium* spp. oocysts were seen in 5.9% (50/848) of samples, of which 43 amplified by PCR-RFLP indicated the following species: *C. hominis*, *C. parvum*, *C. hominis*, *C. parvum*, *C. meleagridis*, and *C. andersoni*. Wider species diversity was found in the rural setting, and may be a result of increased malnutrition and zoonotic exposure in this area [33].

In Kenya, over a year period, a prospective survey on the prevalence of cryptosporidiosis in children less than five years of age was undertaken at six microbiology laboratories on fecal samples submitted for routine parasite and ova investigations [32]. Analysis of 4,899 samples showed cryptosporidiosis prevalence of 4%. Investigations on the nature of enteric diseases prompting ova and cyst examination requests showed 66.4% had acute diarrhoea, 9% had persistent diarrhoea, and 21% had recurrent diarrhoea. The main symptoms were abdominal pain (51.1%), vomiting (51.6%), and abdominal swelling (11%). The prevalence of cryptosporidiosis was highest among children 13 to 24 months of age (5.2%) and least among those

48 to 60 months of age (2%). No significant differences were observed by sex but vomiting was slightly higher in males than in females. Genotype analysis based on polymerase chain reaction-restriction fragment length polymorphism of the 18S rRNA gene fragment showed that 87% (153 of 175) of the *Cryptosporidium* isolates were *C. hominis*, 9% (15 of 175) were *C. parvum*, and remaining 4% were *C. canis*, *C. felis*, *C. meleagridis*, and *C. muris*. The most common protozoa in coinfecting patients were *Entamoeba histolytica*/*E. dispar*, *E. coli*, and *G. intestinalis* (6%, 5%, and 2%, respectively). These results demonstrated that *Cryptosporidium* is among the most common protozoan parasites in children with enteric diseases and that anthroponotic species are the leading cause of human cryptosporidiosis in Kenya, which suggests that human-to-human transmission is the main mode of spread [32].

In the Venda region of South Africa, the prevalence and species distribution of *Cryptosporidium* among schoolchildren and hospital patients was determined [35]. Real time PCR (qPCR) was used for initial screening to detect positive samples, while an 18S rRNA nested PCR followed by restriction fragment length polymorphism was used to determine the species genotype. From a total of 244 stool samples tested, 44 (18%) had *Cryptosporidium* with no significant difference between samples collected from patients attending hospitals 36/197 (18%) and the samples from primary schools 8/47 (17%). The age groups most affected were those from 2 to 5 years old (28.6%) and 50 to 59 years old (50.0%). *Cryptosporidium* was detected in 4 (12.5%) of the 31 HIV positive individuals. Fifty-seven percent of the *Cryptosporidium* positive samples were diarrhoeic and 26 (59.1%) had elevated lactoferrin content. *C. hominis* (82%) was more common than *C. parvum* (18%). This study demonstrated the high prevalence of *Cryptosporidium* infections in the Venda region and its implications in causing diarrhoea and inflammation [35].

In another study, *C. hominis* and *C. parvum* isolates from children in Uganda were characterized by DNA sequence analysis of the GP60 gene [37]. Eight alleles were identified, four *C. hominis* and four *C. parvum*, of which three represented new *C. parvum* families. This data

demonstrated that it is highly likely that the route of transmission is anthroponotic.

Table 2. Examples of *Cryptosporidium* investigations in developing countries which employed molecular methods on *Cryptosporidium* positive human stool samples.

<i>Cryptosporidium</i> species and prevalence	Molecular tools used	Stool sample details	Country	Ref
<i>C. hominis</i> (81%), <i>C. parvum</i> (12%), and <i>C. felis</i> (5.2%)	18S rRNA PCR-RFLP	53 positive samples from children with diarrhoea	India	[28]
<i>C. hominis</i> (87.5%), <i>C. parvum</i> (10%), and <i>C. felis</i> (2.5%)	18S rRNA polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)	40 positive samples from 1,338 examined from diarrheic and non-diarrheic cases	India	[29]
<i>C. hominis</i> (98%), <i>C. meleagridis</i> (4%), and <i>C. felis</i> (2%)	multilocus sequence typing (MLST)	50 positive samples from children	India	[30]
<i>C. hominis</i> (64.6%), <i>C. parvum</i> (18.8%), <i>C. felis</i> (10.4%), <i>C. muris</i> (2.1%), and <i>C. meleagridis</i> (2.1%)	MLST	48 positive samples from human with immunodeficiency virus-infected individuals	India	[31]
<i>C. hominis</i> (87%), <i>C. parvum</i> (9%), <i>C. canis</i> (1.7%), <i>C. felis</i> (1.1%), <i>C. meleagridis</i> (0.6%), and <i>C. muris</i> (0.6%)	18S rRNA PCR-RFLP	183 positive samples from 4,899 fecal samples submitted for routine parasite and ova investigations	Kenya	[32]
<i>C. hominis</i> (48%), <i>C. parvum</i> (18%), <i>C. meleagridis</i> (4%), and <i>C. andersoni</i> (2%)	18S rRNA and COWP gene loci PCR-RFLP	50 positive samples from 848 diarrhoeal stool samples from children aged <five years	Malawia	[33]
<i>C. hominis</i> (95.3%) and <i>C. parvum</i> (4.7%)	MLST	69 positive fecal samples	Malawi	[34]
<i>C. hominis</i> (82%) and <i>C. parvum</i> (18%)	Real time PCR and 18S rRNA nested PCR followed by RFLP	44 positive samples from 244 stool samples tested	South Africa	[35]

In Nepal, a study of acute diarrhoea in 160 children aged five years and below found that *Cryptosporidium* was detected in nine cases (5.6%), and all 50 control children were negative [38]. Another study in western Nepal was conducted to find the association between protozoal agents and persistent diarrhoea in children younger than age 5 years [39]. Stool samples were collected from 253 children with persistent diarrhoea, from 155 children with acute diarrhoea (disease controls) and from 100 healthy

children from the community (normal controls). Of 253 children with persistent diarrhoea, 90 (35.5%) had protozoal infections, 63 (24.9%) helminthic infections, 32 (12.6%) had bacterial infections and 16 had mixed infections. *Giardia lamblia* was the most prevalent (67.7%), followed by *Entamoeba histolytica* (27.7%). HIV infection and severe malnutrition were associated with *Cyclospora cayetanensis* and *Cryptosporidium* spp causing persistent diarrhoea [39].

In Iran, between 2002 to 2005, 15 human and 9 animal stool specimens, collected and diagnosed positive for *Cryptosporidium* by acid-fast staining, were genotyped on the basis of the 18S rRNA gene by nested PCR-restriction fragment length polymorphism (RFLP) and sequencing [9,40]. Isolates of *Cryptosporidium* spp. from human and animal hosts in Iran were characterized on the basis of both the 18S rRNA gene and the Laxer locus [40,41]. *C. hominis*, *C. parvum*, and *C. meleagridis* were all recognized, and zoonotically transmitted *C. parvum* was the predominant species found in humans [40].

An Indian study of 1,338 human stool samples examined from diarrhoeic and non-diarrhoeic cases, *Cryptosporidium* was detected by microscopy in 40 (2.98%) samples, with a prevalence of 4.6% in diarrhoeic cases and 1.2% in non-diarrhoea cases [29]. Molecular characterization of the parasite from diarrhoeic children was carried out by PCR-restriction fragment length polymorphism analysis of the small-subunit (SSU) rRNA gene using nested PCR. At least three genotypes were identified: out of 40 positive samples; of these, 35 were positive for *C. hominis*, four were positive for *C. parvum*, and one was positive for *C. felis*. This study clearly suggested that cryptosporidiosis in this region was caused largely by anthroponotic transmission [29]. In another study in Southern India, genetic characterization of *Cryptosporidium* spp. by PCR-restriction fragment length polymorphism and spatial analysis of cases using Geographical Information Systems technology was conducted for 53 children with cryptosporidial diarrhoea in an urban slum [28]. The two most common species were *C. hominis* (81%) and *C. parvum* (12%). Other species identified were *C. felis* and *C. parvum* (mouse genotype). Five subgenotypes were identified at the Cpgp40/15 locus. Subgenotype Ia predominated among *C.*

hominis isolates, and all *C. parvum* isolates were subgenotype Ic. *C. hominis* infection was associated with a greater severity of diarrhoea. Sequencing of the Cpgp40/15 alleles of *C. felis* and *C. parvum* (mouse genotype) revealed similarities to subgenotype IIa and *C. meleagridis*, respectively. Space-time analysis revealed two clusters of infection due to *C. hominis* Ia, with a peak in February 2005. This study demonstrated space-time clustering of a single subgenotype of *C. hominis* in a setting where cryptosporidiosis is endemic [28]. Gatei *et al.* (2007) collected fifty *Cryptosporidium*-positive specimens from pediatric patients in Kolkata, India, between 2001 and 2004. The specimens were analyzed for parasite genetic structure using multilocus sequence typing (MLST) [30]. Genotype analyses showed the presence of *C. hominis*, *C. meleagridis* and *C. felis* in 49, 2 and 1 patients, respectively (two patients had mixed infections of *C. hominis* and *C. meleagridis*). Muthusamy *et al.* (2006) characterized cryptosporidial infections in 48 human immunodeficiency virus-infected individuals in India by multilocus genotyping. *C. hominis*, *C. parvum*, *C. felis*, *C. muris*, and *C. meleagridis* were identified [31]. Cpgp40/15 PCR-restriction fragment length polymorphism identified six subgenotypes. Cryptosporidial diarrhoea was associated with decreased CD4 counts, below 200 ($P = 0.009$), but not high viral loads.

Ahmad (1995) detected *Cryptosporidium* in eight out of 76 (10.5%) of Malaysian water resources which were examined [42]. In another Malaysian study between July 1994 and January 1995, no *Cryptosporidium* oocysts were detected from two water treatment plants [43]. However, using UK standard methods for isolation and enumeration of cysts and oocysts, *Giardia* cysts were detected in 90% of the raw water samples (range 0-60 cysts per litre). Park *et al.* (2006) identified genotypes of *Cryptosporidium* prevalent among inhabitants and domestic animals (cattle and goats), to elucidate transmittal routes in a known endemic area in Hwasun-gun, Jeollanam-do, Republic of Korea [44]. The existence of *Cryptosporidium* oocysts was confirmed using a modified Ziehl-Neelsen stain. Human infections were found in 7 (25.9%) of 27 people examined. Cattle cryptosporidiosis cases constituted 7 (41.2%) of 17 examined, and goat cases 3 (42.9%) of 7 examined. Species characterizations were

performed on the small subunit of the rRNA gene using both PCR-RFLP and sequence analysis. Most of the human isolates were mixtures of *C. hominis* and *C. parvum* genotypes and similar PCR-RFLP patterns were observed in cattle and goat isolates. However, sequence analyses identified only *C. hominis* in all isolates examined. The natural infection of cattle and goats with *C. hominis* is a new and unique finding in the present study. It was suggested that human cryptosporidiosis in the studied area is caused by mixtures of *C. hominis* and *C. parvum* oocysts originating from both inhabitants and domestic animals [44].

In Libya, between April 2000 to March 2001, standard microbiological techniques were used to examine stool samples from 169 children (70 females) aged a few days to 12 years with acute diarrhoea for viral, bacterial and parasitological agents [45]. A single agent was detected in 44.4% of cases, rotavirus in 26.6%, *Salmonella* in 13.6%, and *Cryptosporidium* in 13% of patients, *Shigella* in 3.6%, *Aeromonas* in 5.5%, *E. histolytica/dispar* in 11.8, and *G. lamblia* in 1.2%. Rotavirus, non-typhoid *Salmonella* and *Cryptosporidium* were the enteric agents most associated with childhood diarrhoea in Zliten [45].

Water and sanitation interventions in developing countries have historically been difficult to evaluate. Steinberg *et al.* (2004) conducted a seroepidemiologic study with multi-target goals [46]. Initially they set out to determine the feasibility of using antibody markers as indicators of waterborne pathogen infection in the evaluation of water and sanitation intervention projects and extended their range of investigations to characterize the epidemiology of waterborne diarrhoeal infections in rural Guatemala, and to measure the age-specific prevalence of antibodies to waterborne pathogens. Sufficient serum was collected from 522 of 590 eligible children divided into six-month age groups for analysis (6-12, 13-18, 19-24, 25-30, and 31-36 months) in 10 study villages. The prevalence of antibodies was lowest in children 6-12 months old compared with the four older age groups for the following pathogens: enterotoxigenic *E. coli* (48%, 81%, 80%, 77%, and 83%), Norwalk virus (27%, 61%, 83%, 94%, and 94%), and *C. parvum* (27%, 53%, 70%, 67%, and 73%). The prevalence of total antibody to hepatitis A virus increased steadily in the three oldest age

groups (40%, 28%, 46%, 60%, and 76%). In contrast, the prevalence of antibody to *Helicobacter pylori* was relatively constant in all five age groups (20%, 19%, 21%, 25%, and 25%). Serology appears to be an efficient and feasible approach for determining the prevalence of infection with selected waterborne pathogens in very young children. Such an approach may provide a suitable, sensitive, and economical alternative to the cumbersome stool collection methods that have previously been used for evaluation of water and sanitation projects in developing countries [46].

An examination of the prevalence of human cryptosporidiosis within the People's Republic of China from 1989-2004 demonstrates its widespread prevalence, particularly in children (Table 3). In addition, these studies indicate that the prevalence of this infection was higher in individuals from rural areas, in comparison to individuals from urban areas. Such a difference as this may reflect the different water treatment processes that are practiced in urban areas compared to rural areas, where effective treatment may be less effective and where there is a greater population density of animals capable of zoonotic transmission. As China is a major producer of horticultural produce, particularly lettuces for home and export markets, and given that lettuce has recently been described as a potential source of *Cryptosporidium* in the food chain, the risk [56] from the transmission of *Cryptosporidium* from contaminated and non-potable water, may be an important source of *Cryptosporidium* both in China and abroad.

Cryptosporidium sp. is a significant cause of diarrhoeal disease, particularly in human immunodeficiency virus (HIV)-infected patients in developing countries. Leav *et al.* (2002) have cloned and sequenced several alleles of the highly polymorphic single-copy *C. parvum* gene Cpgp40/15 [57]. This gene encodes a precursor protein that is proteolytically cleaved to yield mature cell surface glycoproteins gp40 and gp15, which are implicated in zoite attachment to and invasion of enterocytes. The most striking feature of the Cpgp40/15 alleles and proteins is their unprecedented degree of sequence polymorphism, which is far greater than that observed for any other gene or protein studied in *C. parvum* to date. In their study comprising *C. parvum* isolates from

HIV-infected South African children, fifteen isolates exhibited one of four previously identified genotype I alleles at the Cpgp40/15 locus (Ia, Ib, Ic, and Id), while five displayed a novel set of polymorphisms that defined a new Cpgp40/15 genotype I allele, designated genotype Ie.

Table 3. Summary of reported prevalence rates of human cryptosporidiosis in the People's Republic of China (1989-2004).

Year	Geographical Region	Population Examined	Occurrence	Description	Ref
2004	Anhui Province	1204 children from 29 kindergartens	2.35% isolation rate, significantly lower infection rate in urban children (2.14%, 15/684) than the rural ones (5.19%, 27/520).	<i>Cryptosporidium</i> infection was usually subclinical, the major clinical features of cryptosporidium included benign diarrhoea, mild abdominal pain and nausea.	[47]
2002	Yunnan Province	Fresh feces from diarrhoea cases in 7 counties/ cities	5.29% isolation rate, infection rate was high (8.51%) in preschool children and was detected in 6 counties	<i>Cryptosporidium</i> ssp. are widely prevalent in Yunnan Province, particularly in preschool children.	[48]
2002	Anhui Province	5421 fresh stool samples from eleven areas	1.33% (74/5421) <i>C. parvum</i> isolation rate. Among them, the positive rates of oocyst in the areas of Hualbei (1.82%) and Fuyang (1.80%) were higher.	There are two infection peaks in infection of <i>Cryptosporidium parvum</i> and its infection can be found more often in infants, patients with diarrhoea or immunodeficiency, and in rural areas. Subclinical infection is the main manifestation and might be easily misdiagnosed. When the therapeutic effectiveness is low for diarrhoea, the infection of <i>Cryptosporidium parvum</i> should be considered, concerning their age and immune function.	[49]
1997	Changsha	Faecal specimens were collected from 102 outpatients with diarrhoea in Children's Hospital from March to October, 1997	4.90% (5/102) isolation rate. There was no significant difference between females and males (P > 0.05).	Gastrointestinal infection with <i>Cryptosporidium</i> occurred with diarrhoea and in different sex and age groups.	[50]
1984-1993	Hong Kong	214 HIV-1-infected patients, seen between December 1984 and December 1993 in a specialist clinic for HIV/AIDS.	Opportunistic infections among Chinese and non-Chinese were similar in spectrum, though higher frequencies of infection with CMV, <i>Mycobacterium avium intracellulare</i> and tuberculosis were seen among Chinese, whereas the opposite was true for <i>Pneumocystis carinii</i> pneumonia, toxoplasmosis and cryptosporidiosis		[51]

Year	Geographical Region	Population Examined	Occurrence	Description	Ref
1994	Dondian, Linshan, and Fuziyan villages, in rural Anhui Province	A cluster-sampling, cross-sectional study in children less than 16 years of age	0.94% isolation rate, from 320 apparently healthy children less than 10 years of age from Dondian who had stool specimens collected. No positive specimens were found in 239 children studied from Linshan.	These results indicate that cryptosporidial infection is ubiquitous, and is highly endemic in these impoverished communities..	[52]
1989-1992	Xuzhou city and 6 rural areas of Jiangsu Province	6,221 individuals	1.6% isolation rate, <i>Cryptosporidium</i> oocysts were found in 97 of 6,221 individuals as a whole, and were detected in 39 (3.3%) of 1,172 outpatients among the examinees. The incidence was evidently higher in the group of children under 4 than in that aged 4 to 15 (P<0.01).	Diarrhoea, intermittent or persistent, was the main symptom of cryptosporidiosis, being present in 94 (69.1%) of the 136 positive cases, while the other 42 were asymptomatic carriers. The results of routine blood examination and immunoassay performed in one group of the infected children indicated that more than half of them had anemia and lessened cellular immunity. Stool examination of the domestic animals of the affected households showed that a pig and a dog were also positive.	[53]
1992	Xuzhou City and six rural areas of Jiangsu Province	5,089 children (<15 years) were examined	1.75% isolation rate, of the total of 5,089 children examined, 89 were oocyst positive. The incidence was evidently higher in the group of children under age 4 years than it was in children from 4 to 15 years (P<0.01).	Routine blood examination and immunoassay performed on blood samples from some of the infected children indicated that more than half of them had anemia and lower cellular immunity. Diarrhoea was the main symptom of cryptosporidiosis. It was intermittent or persistent and was present in 57 of the 89 children positive for <i>Cryptosporidium</i> oocysts, while the other 32 children were asymptomatic carriers.	[54]
1989	Wuhu, Anhui Province	3,498 paediatric patients taken from five hospitals	1.9% isolation rate; <i>Cryptosporidium</i> oocysts were identified in stool specimens from 67 of 3,498 outpatients. No difference was found between the prevalence of cryptosporidiosis in males and that in females. No <i>Cryptosporidium</i> oocysts were identified in stool specimens from infants under 6 months old.	The prevalence of cryptosporidiosis in rural outpatients was two-fold of that in urban outpatients. The occurrence of cases was highly sporadic, although the prevalence began to rise, where 92.5% of cases were from the last third of June to September. In all the cases no unique characteristic was found in clinical manifestations and stool appearance, and symptoms were resolved spontaneously.	[55]

Surprisingly, only 15 of these isolates exhibited concordant type I alleles at the thrombospondin-related adhesive protein of *Cryptosporidium* and *Cryptosporidium* oocyst wall protein loci, while five isolates (all of which displayed Cpgp40/15

genotype Ic alleles) displayed genotype II alleles at these loci. Furthermore, the last five isolates also manifested chimeric genotype Ic/Ib or Ic/II alleles at the Cpgp40/15 locus, raising the possibility of sexual recombination within and between prototypical parasite genotypes. Lastly, children infected with isolates having genotype Ic alleles were significantly older than those infected with isolates displaying other genotype I alleles.

Detection and diagnosis

Entamoeba histolytica, *G. intestinalis*, and *Cryptosporidium* spp. are not only three of the most important and common diarrhoea-causing parasitic protozoa, but they often have similar clinical presentations [19]. Other opportunistic infections may also induce severe and protracted diarrhoea, including atypical mycobacteria and cytomegalovirus [21]. Ideally diagnosis of diarrhoea should be individually tailored based on presenting symptoms and risk factors. A stepwise diagnostic approach is effective in limiting patient discomfort and minimizing the costs of investigations, starting with microbiologic investigation and proceeding with endoscopy and histology [21].

To obtain valuable epidemiologic data, only identifying *Cryptosporidium* species using conventional criteria, such as oocyst morphology, is inadequate [12]. In the majority of modern clinical laboratories in developed countries, the most widely used staining method for the detection of relatively low numbers of *Cryptosporidium* is still immunofluorescence assay (IFA) [4, 58, 59]. It is often necessary to use an oocyst concentration technique such as immunomagnetic separation (IMS)-recovered oocysts to maintain an acceptable level of assay sensitivity [60,61]. As useful as these laboratory techniques are, they are unable to discriminate between species of *Cryptosporidium* and do not lend themselves to the generation of any useful epidemiological data (Table 4) [59,61,75].

The advent of more specific and sensitive alternative molecular techniques, e.g. nested polymerase chain reaction [PCR], multiplex PCR, and real-time PCR have enabled improved characterization of different species and genotypes of individual *Cryptosporidium* oocysts [12,17,18]. Where possible, molecular methods are useful for rapidly detecting *Cryptosporidium* in

immunocompromised patients and for generating improved and more useful epidemiological data [9,76]. Also, real-time PCR procedures for the detection and genotyping of oocysts of *Cryptosporidium* provide a reliable, specific, and rapid detection method alternative to nested PCR, with a baseline sensitivity of between 1 and 10 oocysts [4,73,77]. Recently, the application of laser-capture microscopy and real-time PCR allows the rapid isolation, detection, and identification of archived environmental slides and clinical slides, unlocking genetic information [78]. However, the relatively high cost of molecular methods at present has limited their application in developed and developing countries [19]. The methodologies used in the detection of *Cryptosporidium*-specific antibodies vary widely, which complicates comparison of results [11]. The use of the recombinant CP41 antigen in a standardized serodiagnostic assay could provide a reliable and cost-effective method for assessing human exposure to *Cryptosporidium* in developing countries [11].

Disease treatment and prevention

Malnutrition, immunosuppression, young age and an increase in the preceding diarrhoea burdens are all risk factors for the development of persistent diarrhoea [16]. Patient management strategies include rehydration, adequate diet, micronutrient supplementation, and antimicrobials [16]. Persistent diarrhoea seriously affects nutritional status, growth, and intellectual function. Meeting these challenges is profoundly important, particularly in developing countries. Aggressive treatment of infectious diarrhoea is required in severely immunocompromised children [23]. However, antiretroviral therapy prevents the development of severe cryptosporidiosis in HIV-infected persons.

The United States Food and Drug Administration recently approved the use of nitazoxanide for treatment of diarrhoea caused by *Giardia* or *Cryptosporidium* spp. [79, 80]. This novel agent has a broad spectrum of activity against many other gastrointestinal pathogens, including bacteria, roundworms, flatworms, and flukes.

Nitazoxanide is used in many areas of the world, especially in Central and South America, as a broad-spectrum agent in adults and children

because it appears to be well tolerated, it has a relatively low incidence of adverse effects, and it displays no significant known drug-to-drug interactions [80]. However, it is not effective against cryptosporidiosis in immunocompromised persons.

In its most recent edition of Guidelines for Drinking-Water Quality (2006) (GDWQ) the World Health Organization (WHO) promoted the use of risk assessment coupled with risk management for the control of water-borne pathogens in drinking water supplies [81].

Table 4. Description of common methods used for the detection of *Cryptosporidium* spp. [4]*.

Technique	Principle	Sensitivity	Advantages/Disadvantages	Ref
Modified acid fast stain	Staining of cryptosporidia by a modified Ziehl Neelsen or other technique.	10-fold lower than IFA	+ Simple and can be used for range of other parasites. - Non specific	[62]
Immunofluorescent staining (IFA)	Anti- <i>Cryptosporidium</i> specific fluorescent antibody stain	10,000-50,000 oocysts/gram of feces	+ Highly specific +With morphological verification - Mo viability assessment	[62, 63]
Enzyme immunoassay (EIA)	<i>Cryptosporidium</i> antigen capture ELISA	Less sensitive than IFA	+Specific and rapid -Detect antigens of developmental stages -Without morphological verification	[64]
Immunochromatography	<i>Cryptosporidium</i> antigen capture colorimetric assays	Less sensitive than IFA	+Rapid -Prone to QA/QC problems	[64]
Fluorescent <i>in-situ</i> hybridization (FISH)	<i>In-situ</i> hybridization using fluorescent-labeled complementary DNA oligonucleotide probes		+ Highly specific - RNA can persist after cell death	[65]
Nested PCR	Two-step PCR using two sets of primers. Common gene targets are gp60, hsp70, 18S rRNA, COWP and TRAP (C1 and C2)	1 oocyst	+ Highly sensitive and specific detection of <i>Cryptosporidium m.</i> - Sequencing or RFLP analysis is required to identify species/genotypes - Relatively long procedure	[9]
PCR-RFLP	Restriction analysis of PCR product after amplification of genomic DNA	1 oocyst	+ Can distinguish between most of the <i>Cryptosporidium</i> species and genotypes - Relatively long procedure compared to real time PCR	[9, 66]

Real-time PCR	Real time detection of DNA using hybridisation probes	1 oocyst	+ Rapid, highly sensitive and specific detection. + Could be used as routine in-line detection + Can be used for species differentiation - Expensive equipment	[67, 68]
Microsatellite analysis	PCR amplification of microsatellites, which serve as polymorphic markers.	Requires further investigation	+ Can distinguish between isolates of same species + Does not require further analysis	[69, 70]
Technique	Principle	Sensitivity	Advantages/Disadvantages	Ref
Real time RT-PCR	Real time detection of mRNA	Depends on gene copy number	+ Rapid, highly sensitive technique + Can potentially be used as viability assay - Currently not robust enough to be used as routine diagnostic tool	[70, 73]
Nucleic acid sequence-based amplification (NASBA)	NASBA is an isothermal amplification method which uses single-stranded RNA as a template, single stranded complementary RNA being amplified in the course of the reaction.	5 oocysts	+ Potential ability to detect viable-only oocyst by amplification of RNA samples. + Does not require a thermocycler. - Requires further development for use as diagnostic tool	[74]

+ : advantage, - : disadvantage

Quantitative microbial risk assessment (QMRA) provides a tool for estimating the disease-burden from pathogenic microorganisms in water, using information about the distribution and occurrence of the pathogen or an appropriate surrogate [82]. This information may then be used to formulate appropriate management practices for the water supply system. Although QMRA has been used to estimate disease burden from water supplies in developed countries, the method has not been evaluated in developing countries where relevant data may be scarce [82]. QMRA could be used increasingly in countries with limited data, and the outcome can provide valuable information for the management of water supplies. Because of the possible transmission of *C. canis* among children and dogs in households [83], improvements in water, sanitation, household hygiene and animal control are required to reduce the incidence of infection in this population [33].

Florén *et al.* (2006) conducted a study at Braithwaite Memorial Specialist Hospital, Port

Harcourt, Nigeria, on thirty patients with HIV-associated diarrhoea [84]. HIV-associated diarrhoea occurs in nearly all patients with acquired immunodeficiency syndrome (AIDS) in the developing countries. Diarrhoea is caused by the HIV-related immune dysfunction and is pivotal in the decrease of the helper T-cell (CD4 +) population. Enteric pathogens in HIV-associated diarrhoea are, for example, *Cryptosporidium*, amoeba and *Campylobacter* species. Bovine colostrum is the first milk the suckling calf receives from the cow. It is rich in immunoglobulins, growth factors, antibacterial peptides and nutrients. It supplies the calf with a passive immunity before its own active immunity is established. ColoPlus is a product based on bovine colostrums. As well as having a high nutritional value, it is designed for slow passage through the gastrointestinal tract. In the study by Florén *et al.* (2006) the patients were treated with ColoPlus for 4 weeks in an open-labelled non-randomized study, after an observational period of one week. After a post-treatment period of another two weeks, treatment with anti-HIV drugs was started, if deemed appropriate. The effects on the frequency of stool evacuations per day, on body-weight, fatigue, haemoglobin levels and CD4+ counts before (week 1) and after treatment with ColoPlus (week 7) were measured. There was a dramatic decrease in stool evacuations per day from 7.0+/-2.7 to 1.3+/-0.5 (+/-SD), a substantial decrease in self-estimated fatigue of 81%, an increase in body-weight of 7.3 kg per patient and an increase in CD4+ count by 125%. ColoPlus may be an important alternative or additional treatment in HIV-associated diarrhoea [84].

Conclusions

The prevalence of *Cryptosporidium* in humans in both developed and developing countries demonstrates the magnitude of this parasite in public health [85]. The high occurrence of *Cryptosporidium* in surface water sources underlines the need for frequent and improved monitoring of the parasite in drinking water sources [86]. Better education and increased awareness of cryptosporidiosis by the general public could potentially reduce case numbers [87]. Improved and more economically viable detection methods with the ability to differentiate species in the assessment of infection and identification of

sources of contamination are vital, and would provide important data on the levels of disease burden due to zoonotic transmission [88].

The roles of humans, livestock and wildlife in the transmission of *Cryptosporidium* remain largely unclear. The continued monitoring (using appropriate molecular methods) of *Cryptosporidium* in surface water, livestock, wildlife and humans will increase our knowledge of infection patterns and transmission of *Cryptosporidium* in the future [88]. Proteomic profiling is a useful approach for obtaining a global overview of the proteins present in a system under differing conditions and can aid in understanding the molecular determinants involved with pathogenesis and vaccine development [90].

Until recently, compared with other Apicomplexans, e.g. *Toxoplasma gondii*, both the limited supply of purified parasite material and the lack of transfection systems have restricted analyses of proteins in parasites of the genus *Cryptosporidium* [90]. Snelling *et al.* (2007) discovered eight *Cryptosporidium* and Apicomplexan-specific proteins which augmented significantly during excystation. These eight proteins may represent promising targets for developing vaccines or chemotherapies that could block parasite entry into host cells [90]. Encouragingly, Apicomplexan proteins, including those from *Plasmodium*, *Cryptosporidium* and *Toxoplasma*, can be expressed, purified and crystallized using established high throughput methods [91,92]. Specifically, it has been demonstrated that *E. coli* is indeed an effective heterologous expression platform for these proteins, even for Plasmodium genes with extreme AT-biases [91,92]. Proteins from *C. parvum* can be expressed in *E. coli* much more readily, likely due to noticeably lower frequency of low complexity regions and signal peptides [92]. These recent developments should help with future *Cryptosporidium* vaccine and chemotherapeutic development.

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