CHAPTER 4

Cryptosporidium and Cryptosporidiosis

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4.1 PREFACE

Cryptosporidium spp. are apicomplexan parasites that inhabit the brush-borders of the gastrointestinal epithelium (Bird and Smith, 1980). Initially thought to be only a pathogen of young animals such as calves, lambs, piglets, and foals, cryptosporidiosis is now known to be an important cause of enterocolitis, diarrhea, and cholangiopathy in humans (Current *et al.*, 1983). Several *Cryptosporidium* spp. are now recognized to infect humans and more to infect other vertebrates (Xiao *et al.*, 2004a). Healthy children and adults and young animals with cryptosporidiosis usually have a short-term illness accompanied by watery diarrhea, vomiting, malabsorption, and weight loss. In humans and animals with immunodeficiencies, and snakes, however, the infection can be protracted and life-threatening (Hunter and Nichols, 2002).

Cryptosporidium oocysts are environmentally resistant, retain their infectious potential for considerable time in moist environments, such as water, soil, fresh seafood and produce (Rose, 1997), and survive most water disinfection treatments as well (Korich *et al.*, 1990). Two important fecal-oral transmission routes include direct contact with infected persons (person-to-person or anthroponotic transmission) or animals (zoonotic transmission), and consumption of contaminated water (waterborne transmission) or food (foodborne transmission). Thus, *Cryptosporidium* spp. are well recognized water and food-borne pathogens, having caused many outbreaks of human diarrheal disease in the United States and other developed countries (Anonymous, 1984; Current *et al.*, 1983; D'Antonio *et al.*, 1985; Joce *et al.*, 1991; MacKenzie *et al.*, 1994b; Millard *et al.*, 1994). Water and food probably also play an important role in the transmission of cryptosporidiosis in endemic areas, even though the disease burden attributable to them is not fully clear.

4.2 TAXONOMY

Cryptosporidium spp. belong to the family Cryptosporidiidae, which is a member of the phylum Apicomplexa. The exact placement of Cryptosporidiidae in Apicomplexa is uncertain. It was long considered a member of the class Coccidea, in the order of Eimeriida or Eucoccidiorida (Corlis, 1994). Recent phylogenetic studies, however, indicate that *Cryptosporidium* spp. are more related to gregarines than to coccidia (Carreno *et al.*, 1999). Extra-celluar gregarine-like reproductive stages have been described in *Cryptosporidium andersoni* and *Cryptosporidium parvum* (Hijjawi *et al.*, 2002). Thus, *Cryptosporidium* spp. are no longer considered coccidian parasites.

Cryptosporidium spp. were first recognized by Tyzzer in 1907, who described *Cryptosporidium muris* in the stomach of laboratory mice (Tyzzer, 1907, 1910). Later in 1912, Tyzzer described a second species in laboratory mice, *C. parvum* (Tyzzer, 1912). This new species differed from *C. muris* not only by infecting the small intestine instead of the stomach, but also by having smaller oocysts, the environmentally robust stage of the parasite (Upton and Current, 1985).

Over the next 50 years following the initial description of *Cryptosporidium*, these parasites were commonly confused with sporocysts of *Sarcocystis*. Several new *Cryptosporidium* species were described during the period, mostly based on sporocysts of *Sarcocystis* spp. Subsequently, it was thought that because *Cryptosporidium* was closely related to *Eimeria, Cryptosporidium* spp. also could not normally be transmitted from one species of animals to another (Levine, 1980). This erroneous concept of strict host specificity led to the description and report of multiple new species during the 1960–1980s, which are no longer considered valid, such as *Cryptosporidium anserinum* in geese (Proctor and Kemp, 1974), *Cryptosporidium agni* in sheep (Barker and Carbonell, 1974), *Cryptosporidium bovis* in neonatal calves (Barker and Carbonell, 1974), *Cryptosporidium rhesi* in monkeys (Levine, 1980), and *Cryptosporidium cuniculus* in rabbits (Inman and Takeuchi, 1979).

Infection and cross-transmission studies conducted in the 1970s and 1980s demonstrated that *Cryptosporidium* isolates could indeed frequently be transmitted from one host species to another (Tzipori *et al.*, 1981a, 1981b, 1982). These findings led to the synonymization of many species into *C. parvum*, and were the basis for proposing the monospecific structure of the genus *Cryptosporidium*. As a result, *C. parvum* was used extensively for the description of *Cryptosporidium* spp. from most mammals including humans (Tzipori *et al.*, 1980; Upton and Current, 1985).

The recent use of molecular methods in the characterization of *Cryptosporidium* has helped to resolve existing confusions in the taxonomy of this genus (Fayer *et al.*, 2000a; Morgan *et al.*, 1999b; Xiao *et al.*, 2000b, 2004a). These molecular tools have been very valuable when used in conjunction with morphological, biological, or host specificity studies. This has resulted in the validation of several *Cryptosporidium* described earlier, such as *Cryptosporidium meleagridis* in birds, *Cryptosporidium wrairi* in guinea pigs, and *Cryptosporidium felis* in cats. It is now well known that various *Cryptosporidium* isolates do have differences in host specificity, but one *Cryptosporidium* sp. usually infect a limited spectrum of animals, especially if the host animals are related. This new *Cryptosporidium* taxonomic paradigm has also led to the establishment of several new *Cryptosporidium* species, such as *Cryptosporidium hominis* (previously known as *C. parvum* genotype 1 or the human genotype) in humans, *C. andersoni* (previously known as *C. muris*-like or *C. muris* bovine genotype) and *C. bovis* (previously known as *Cryptosporidium* bovine genotype B) in weanling calves and adult cattle, *Cryptosporidium canis* (previously known as *C. parvum* dog genotype) in dogs, and *Cryptosporidium suis* (previously known as *Cryptosporidium* pig genotype I) in pigs. Now, there are 15 established *Cryptosporidium* species in fish, reptiles, birds, and mammals (Table 4.1). There are also many host-adapted *Cryptosporidium* genotypes that do not yet have designed species names because of the lack of morphologic and biologic characterizations,

Table 4.1. Currently recognized *Cryptosporidium* species.

Species	*Major host*	*Minor host*	*Infection site*	*Reference*
C. andersoni	Cattle, bactrian camels	Sheep	Stomach	(Lindsay *et al.*, 2000)
C. baileyi	Chicken, turkeys	Cockatiels, ducks, ostriches, quails	Intestine, respiratory track, bursa	(Current *et al.*, 1986)
C. bovis	Cattle, yaks	Sheep	Intestine	(Fayer *et al.,* 2005)
C. canis	Dogs, foxes, wolves	Humans	Intestine	(Fayer *et al.*, 2001)
C. felis	Cats	Humans, cattle	Intestine	(Iseki, 1979)
C. galli	Chickens, finches, capercalles, grosbeaks		Proventriculus	(Ryan *et al.*, 2003)
C. hominis	Humans, monkeys	Sheep, dugongs	Intestine	(Morgan-Ryan *et al.*, 2002)
C. meleagridis	Turkeys, humans	Parrots	Intestine	(Slavin, 1955)
C. molnari	Fish		Stomach	(Alvarez-Pellitero and Sitja-Bobadilla, 2002)
C. muris	Rodents, bactrian camels	Humans, rock hyrax, mountain goats	Stomach	(Tyzzer, 1910)
C. parvum	Cattle, sheep, goats, deer, humans	Mice, pigs, horses	Intestine	(Upton and Current, 1985)
C. saurophilum	Lizards	Snakes	Intestine	(Koudela and Modry, 1998)
C. serpentis	Snakes, lizards		Stomach	(Tilley *et al.*, 1990)
C. suis	Pigs	Humans	Intestine	(Ryan *et al.*, 2004b)
C. wrairi	Guinea pigs		Intestine	(Vetterling *et al.*, 1971)

such as *Cryptosporidium* horse, rabbit, mouse, ferret, deer mouse, skunk, squirrel, bear, deer, deer-like, cervine, fox, mongoose, wildebeest, duck, woodcock, snake, tortoise, goose I and II, muskrat I and II, opossum I and II, marsupial I and II, and pig II genotypes (Xiao *et al.*, 2004a).

Currently, eight *Cryptosporidium* spp. have been reported in humans: *C. hominis*, *C. parvum*, *C. meleagridis*, *C. felis*, *C. canis*, *C. muris*, *C. suis*, and *Cryptosporidium* cervine genotype. Humans are most frequently infected with *C. hominis* and *C. parvum*. The former almost exclusively infects humans, thus is considered an

anthroponotic parasite, whereas the latter infects both humans and domestic and wild ruminants, thus is considered a zoonotic pathogen. The contribution of the two species to human cryptosporidiosis differs among geographic areas, with *C. parvum* responsible for more infection than *C. hominis* in Europe and Kuwait, and *C. hominis* responsible for most human infections in the rest of the world. Other species, such as *C. meleagridis*, *C. felis*, and *C. canis*, are less common. In contrast, *C. muris*, *C. suis*, and *Cryptosporidium* cervine genotypes have been found only in a few human cases (Xiao *et al.*, 2003, 2004a; Xiao and Ryan, 2004). Despite earlier suggestion that unusual zoonotic species usually infect immunocompromised persons, a recent study in Peru suggests that there is no significant difference in the distribution of *Cryptosporidium* species between AIDS patients and children living in the same geographic area (Cama *et al.*, 2003).

4.3 LIFE CYCLE AND DEVELOPMENTAL BIOLOGY

Cryptosporidium spp. are intracellular parasites that primarily infect epithelial cells of the stomach or intestine. The infection site varies according to species, but almost the entire development of *Cryptosporidium* spp. occur between the two lipoprotein layers of the membrane of the epithelial cells (Bird and Smith, 1980), with the exception of *Cryptosporidium molnari*, for which oogonial and sporogonial stages are located deeply within the epithelial cells (Alvarez-Pellitero and Sitja-Bobadilla, 2002; Ryan *et al.*, 2004a). *Cryptosporidium* infections in humans or other susceptible hosts start with the ingestion of viable oocysts, the infectious stage that is environmentally resistant. Upon gastric and duodenal digestion, four sporozoites are liberated from each excysted oocyst, invade the epithelial cells, and develop into trophozoites surrounded by a parasitophorous vacuole. Within the epithelial cells, trophozoites undergo several generations of asexual amplification called merogony, leading to the formation of different types of meronts. The types of meronts depend on *Cryptosporidium* species. For *C. parvum*, there are two types of meronts. The type 1 meront develops six to eight nuclei, giving rise to six to eight merozoites. These stages are morphologically similar to sporozoites and can infect neighboring epithelial cells, forming more type 1 meronts or the new type 2 meronts. The latter develop four nuclei, forming four merozoites. As with type 1 merozoites, these merozoites are released and infect new cells to generate more type 2 meronts, or can differentiate into sexually distinct stages called macro- and micro-gametocytes in a process called gametogony. New oocysts, formed in the epithelial cells from the fusion of macro-gametocytes and micro-gametes, are sporulated *in situ* in a process called sporogony, and contain four sporozoites. It is believed by some that about 20% are "thin walled" and may excyst within the digestive tract of the host, leading to the infection of new cells (autoinfection). The remaining 80% of oocysts are excreted into the environment, are resistant to low temperature, high salinity, and most disinfectants, and can initiate infection in a new host upon ingestion. Thus, the only extracellular stages in the *Cryptosporidium* life cycle are released sporozoites, merozoites, and microgametes, which are briefly in the lumen of the digestive tract (Fayer *et al.*, 1997). However, recently, a gregarine-like stage has been described in

C. andersoni and *C. parvum*, which undergo multiplication through syzgy, a sexual reproduction process involving the end-to-end fusion of two or more parasites (Hijjawi *et al.*, 2002). If verified by others, this would have major implications in our understanding of the *Cryptosporidium* biology, genetics, and transmission.

Like other members of the Apicomplexa, sporozoites, and merozoites of *Cryptosporidium* use the apicomplex for invasion. Unlike other apicomplexan parasites, *Cryptosporidium* spp. have no polar rings and the conoid as part of the apicomplex, with only a relict mitochondrion, no sporocysts and plastids, and no flagelles in micro-gametes. At the contact site between host cells and *Cryptosporidium* developmental stages, there is also a unique electron-dense attachment or feeder organelle, which is supposedly involved in selective transport of nutrients from host cells into developing parasites. The prepatent period (time from ingestion of infective oocysts to the completion of endogenous development and excretion of new oocysts) varies with species, hosts, and infection doses. This is usually between 4 and 14 days.

4.4 EPIDEMIOLOGY AND TRANSMISSION

4.4.1 Cryptosporidiosis in Immunocompetent Persons

In developing countries, human *Cryptosporidium* infection occurs mostly in children younger than five-years old, with peak occurrence of infections and diarrhea in children less than 2 years of age (Bern *et al.*, 2000, 2002; Bhattacharya *et al.*, 1997; Mata, 1986; Newman *et al.*, 1999). Frequent symptoms include diarrhea, abdominal cramps, vomiting, headache, fatigue, and low-grade fever (Nimri and Hijazi, 1994). The diarrhea can be voluminous and watery, but usually resolves within one to two weeks without treatment. Not all infected children have diarrhea or other gastrointestinal symptoms, and the occurrence of diarrhea in children with cryptosporidiosis can be as low as 30% in community-based studies (Bern *et al.*, 2002; Xiao *et al.*, 2001a). Even subclinical cryptosporidiosis exerts a significant adverse effect on child growth, as infected children with no clinical symptoms experience growth faltering, both in weight and in height (Checkley *et al.*, 1997, 1998). *Cryptosporidium*-infected children may never have enough catch-up growth covered for the growth retardation (Checkley *et al.*, 1998; Molbak *et al.*, 1997). Children can have multiple episodes of cryptosporidiosis, implying that the anti-*Cryptosporidium* immunity in children acquired is short-lived or incomplete (Bern *et al.*, 2000, 2002; Newman *et al.*, 1999; Xiao *et al.*, 2001a;). Cryptosporidiosis has been associated with increased child mortality in developing countries (Tumwine *et al.*, 2003).

In developed countries, *Cryptosporidium* infection occurs later in life of children than in developing countries, probably due to later exposures to contaminated environments as a result of better hygiene. In a study conducted in Kuwait, the median age of children with cryptosporidiosis was 4.5 years (Sulaiman *et al.*, 2005). Children in these countries frequently acquire *Cryptosporidium* infection from another infected child attending the same daycare or school, probably via person-to-person transmissions (Alpert *et al.*, 1984; Lacroix *et al.*, 1987; Tangermann *et al.*, 1991; Taylor *et al.*, 1985). Cryptosporidiosis is also common in the elderly in nursing

homes, where person-to-person transmission probably also plays a major role in the spread of *Cryptosporidium* infections (Neill *et al.*, 1996). In rural areas, zoonotic infections via direct contact with farm animals have been reported many times, but the relative importance of direct zoonotic transmission of cryptosporidiosis is not entirely clear (Current *et al.*, 1983; Miron *et al.*, 1991). In the general population, a substantial number of adults are probably susceptible to *Cryptosporidium* infection, as sporadic infections occur in all age groups in the United States and United Kingdom, and traveling to developing countries and consumption of contaminated food or water can frequently lead to infection (Dietz and Roberts, 2000; Dietz *et al.*, 2000; Goh *et al.*, 2004; Roy *et al.*, 2004). Hemodialysis patients with chronic renal failure are also frequently infected with *Cryptosporidium* (Chieffi *et al.*, 1998; Turkcapar *et al.*, 2002).

Unlike in developing countries, immunocompetent persons with sporadic cryptosporidiosis in industrialized nations usually have diarrhea (Anonymous, 1990; Assadamongkol *et al.*, 1992; Chmelik *et al.*, 1998; Daoud *et al.*, 1990; Goh *et al.*, 2004; Robertson *et al.*, 2002a; Thomson *et al.*, 1987). The median number of stools per day during the worst period of the infection is 7–9.5 in Australia (Robertson *et al.*, 2002a). The durations of illness are a mean of 12 days in Finland, and a median of 9 days in the United Kingdom and 15–21 days in Australia (Goh *et al.*, 2004; Jokipii and Jokipii, 1986; Robertson *et al.*, 2002a), with a median of 5 days off work or study (Robertson *et al.*, 2002a). Other common symptoms include abdominal pain (in 72.4–91.7% patients), vomiting (in 55.2–70.9% patients), and low-grade fever (in 38.1–48.5% patients) (Goh *et al.*, 2004; Jokipii and Jokipii, 1986; Robertson *et al.*, 2002a). In the United States, United Kingdom, and Australia, 14.4–17.4%, 8.5–22.1%, and 7–11.9% patients with sporadic cryptosporidiosis require hospitalization, respectively (Dietz *et al.*, 2000; Goh *et al.*, 2004; Robertson *et al.*, 2002a).

4.4.2 Cryptosporidiosis in Immunocompromised Persons

Cryptosporidiosis is common in immunocompromised persons, such as AIDS patients, persons with primary immunodeficiency, and cancer and transplant patients undergoing immunosuppressive therapy (Heyworth, 1996; Hunter and Nichols, 2002; McLauchlin *et al.*, 2003). It is frequently associated with chronic, life-threatening diarrhea (Flanigan *et al.*, 1992; Heyworth, 1996; Hunter and Nichols, 2002). In HIV+ persons, the occurrence of cryptosporidiosis increases as the CD4+ lymphocyte cell counts fall, especially below 200 cells/:l (Flanigan *et al.*, 1992; Navin *et al.*, 1999; Pozio *et al.*, 1997). Manabe *et al.* (1998) described four clinical syndromes of cryptosporidiosis in the United States: chronic diarrhea (36% of patients), cholera-like disease (33%), transient diarrhea (15%), and relapsing illness (15%). Sclerosing cholangitis and other biliary involvements, however, are also very common in AIDS patients with cryptosporidiosis (Chen and LaRusso, 2002; French *et al.*, 1995; Hashmey *et al.*, 1997; McGowan *et al.*, 1993; Teixidor *et al.*, 1991; Vakil *et al.*, 1996). Symptoms of cryptosporidiosis in AIDS patients vary in severity, duration, and responses to drug treatment (Flanigan and Graham, 1990; Goodgame *et al.*, 1993; Manabe *et al.*, 1998; McGowan *et al.*, 1993). Much of this variation can be explained by the degree of immunosuppression (Flanigan *et al.*, 1992; McGowan *et al.*, 1993). In addition, variation in the infection site (gastric infection, proximal

small intestine infection, ileo-colonic infection, versus pan-enteric infection) has been seen in AIDS patients with cryptosporidiosis (Clayton *et al.*, 1994; Kelly *et al.*, 1998; Lumadue *et al.*, 1998; Ventura *et al.*, 1997), and this anatomic variation may also contribute to differences in disease severity and survival (Clayton *et al.*, 1994; Lumadue *et al.*, 1998). Cryptosporidiosis in AIDS patients is associated with increased mortality and shortened survival (Colford *et al.*, 1996; Manabe *et al.*, 1998)

4.4.3 Transmission Routes and Infection Sources: Anthroponotic Versus Zoonotic Transmission

Cryptosporidium infections normally start with the ingestion of infectious oocysts. This parasite has a worldwide distribution and is ubiquitously present in the environment. Humans can acquire *Cryptosporidium* infections through several transmission routes (Clark, 1999; Griffiths, 1998), such as direct contact with infected persons or animals, and consumption of contaminated water (drinking or recreational) or food. However, the relative role of each in the occurrence of *Cryptosporidium* infection in humans is unclear. Several studies in the United States and Europe have shown that cryptosporidiosis was more common in homosexual men than persons with other HIV-transmission categories (Hashmey *et al.*, 1997; Hellard *et al.*, 2003; Soave *et al.*, 1984), indicating that direct person-to-person or anthroponotic transmission of cryptosporidiosis is common. Contact with persons with diarrhea has been identified as a major risk factor in sporadic *Cryptosporidium* infections in the United States, United Kingdom, and Australia (Hunter *et al.*, 2004b; Robertson *et al.*, 2002a; Roy *et al.*, 2004).

Shortly after the discovery of cryptosporidiosis in humans it has been found that humans can acquire *Cryptosporidium* infection via contact with infected farm animals (Current *et al.*, 1983). However, only a few case control studies assessed the role of zoonotic transmission in the acquisition of cryptosporidiosis in humans. In the United States, United Kingdom, and Australia, contact with farm animals is a major risk factor in the sporadic cases of human cryptosporidiosis (Goh *et al.*, 2004; Hunter *et al.*, 2004b; Robertson *et al.*, 2002a; Roy *et al.*, 2004). Contact with pigs, dogs, or cats is also a risk factor for cryptosporidiosis in children in Guinea-Bissau and Indonesia, (Katsumata *et al.*, 1998; Molbak *et al.*, 1994), but this is actually a protective factor in Australia (Robertson *et al.*, 2002a). A weak association was observed between the occurrence of cryptosporidiosis in HIV+ persons and contact with dogs, but not other animals (Glaser *et al.*, 1998). In other studies, no increased risk in the acquisition of cryptosporidiosis was associated with contact with animals (Nchito *et al.*, 1998; Pereira *et al.*, 2002a).

The distribution of *C. parvum* and *C. hominis* in humans is probably a good indicator of the transmission routes. Thus far, studies conducted in tropical countries such as Peru, Thailand, Malawi, Uganda, Kenya, and South Africa showed a dominance of *C. hominis* in children or HIV+ adults (Gatei *et al.*, 2003; Leav *et al.*, 2002; Peng *et al.*, 2003a; Tiangtip and Jongwutiwes, 2002; Tumwine *et al.*, 2003; Xiao *et al.*, 2001a). In Europe, however, several studies have shown a slightly higher prevalence of *C. parvum* than *C. hominis* in both immunocompetent and immunocompromised persons (Alves *et al.*, 2003b; Chalmers *et al.*, 2002; Guyot

et al., 2001; McLauchlin *et al.*, 2000). In contrast, Kuwaiti children were almost exclusively infected with *C. parvum* (Sulaiman *et al.*, 2005). The differences in the distribution of *Cryptosporidium* genotypes in humans is considered an indication of differences in infection sources (Learmonth *et al.*, 2001, 2004; McLauchlin *et al.*, 2000); the occurrence of *C. hominis* in humans is most likely due to anthroponotic transmission, whereas the predominance of *C. parvum* in a population has been considered the result of zoonotic transmission. Thus, in most tropical countries, it is possible that anthroponotic transmission of *Cryptosporidium* play a major role in human cryptosporidiosis; whereas in Europe, both anthroponotic and zoonotic transmissions are important. Indeed, in areas with a high percentage of infections due to *C. parvum*, massive slaughtering of farm animals during foot and mouth disease outbreaks can result in a reduction in the proportion of human infections due to *C. parvum* (Hunter *et al.*, 2003; Smerdon *et al.*, 2003).

Nevertheless, recent subtyping studies have shown that not all *C. parvum* infections in humans are results of zoonotic transmission (Alves *et al.*, 2003b; Mallon *et al.*, 2003b; Xiao *et al.*, 2003). Among the *C. parvum* GP60 subtype families identified, alleles IIa and IIc (previously known as Ic) are the two most common ones. The former has been identified in both humans and ruminants, thus serving as a zoonotic pathogen, whereas the latter has only been seen in humans (Alves *et al.*, 2003b; Peng *et al.*, 2003b; Xiao *et al.*, 2003), thus serving as an anthroponotic pathogen. In Lima, Peru, all *C. parvum* infection in children and HIV+ persons are due to the subtype family IIc, indicating that anthroponotic transmission of *C. parvum* is common in certain areas (Xiao *et al.*, 2004a). Even in the United Kingdom where zoonotic transmission is known to play a significant role in the transmission of human cryptosporidiosis, anthroponotic transmission of *C. parvum* is also common (Mallon *et al.*, 2003a).

4.4.4 Waterborne Transmission

Epidemiologic studies have frequently identified water as a major route of *Cryptosporidium* transmission in disease-endemic areas (Gallaher *et al.*, 1989; Nimri and Hijazi, 1994; Weinstein *et al.*, 1993). In most tropical countries, *Cryptosporidium* transmission in children is usually associated with the rainy season, and waterborne transmission is considered a major route in epidemiology of cryptosporidiosis in these areas (Bern *et al.*, 2000; Bhattacharya *et al.*, 1997; Javier Enriquez *et al.*, 1997; Katsumata *et al.*, 1998; Moodley *et al.*, 1991; Nath *et al.*, 1999; Newman *et al.*, 1999; Peng *et al.*, 2003a; Perch *et al.*, 2001; Tumwine *et al.*, 2003). However, some studies have failed to show a direct linkage between seasonal incidence of cryptosporidiosis and rainfall (Bern *et al.*, 2002).

Seasonal variations in the incidence of human *Cryptosporidium* infection in industrialized nations have also been attributed to waterborne transmission (Brandonisio *et al.*, 1999; Dietz and Roberts, 2000; Dietz *et al.*, 2000; McLauchlin *et al.*, 2000; Roy *et al.*, 2004). In the United States, there are two annual peaks in the number of cryptosporidiosis cases in HIV+ persons: one in spring and one in late summer (Inungu *et al.*, 2000; Sorvillo *et al.*, 1998). In the general population, there is also an annual late summer peak in sporadic cases of cryptosporidiosis (Dietz and Roberts, 2000; Roy *et al.*, 2004). It is generally accepted that the late summer peak of

cryptosporidiosis cases is due to recreational activities such as swimming and water sports, suggesting that waterborne transmission may be important in cryptosporidiosis epidemiology. Nevertheless, seasonal transmission of cryptosporidiosis in HIV+ persons is not always associated with rainfall (Sorvillo *et al.*, 1998).

The role of drinking water in sporadic *Cryptosporidium* infection is not clear. In Mexican children living near the United States border, cryptosporidiosis is associated with consumption of municipal water instead of bottled water (Leach *et al.*, 2000). In England, the number of glasses of tap water drunk at home each day is associated with sporadic cases of cryptosporidiosis (Hunter *et al.*, 2004b). In the United States, drinking untreated surface water was identified as a risk factor for the acquisition of *Cryptosporidium* in a small case control study (Gallaher *et al.*, 1989). Residents living in cities with surface-derived drinking water generally have higher blood antibody levels against *Cryptosporidium* antigens than those living in cities with ground water as drinking water, indicating drinking water plays a role in the transmission of human cryptosporidiosis (Frost *et al.*, 2001, 2002, 2003). An earlier study in South Australia also showed an association between consumption of spring water or main water rather than rain water, and the occurrence of cryptosporidiosis (Weinstein *et al.*, 1993). A more recent study in the same area, however, suggested that waterborne transmission in the area was mainly due to swimming in public pools and consumption of unboiled rural water rather than consumption of tap water (Robertson *et al.*, 2002a). Case control studies conducted in both immunocompetent persons and AIDS patients in the United States also have failed to show a direct linkage of *Cryptosporidium* infection to drinking water (Khalakdina *et al.*, 2003; Sorvillo *et al.*, 1994).

Numerous waterborne outbreaks of cryptosporidiosis have occurred in the United States, Canada, United Kingdom, France, Australia, Japan, and other industrialized nations (Dalle *et al.*, 2003; Lemmon *et al.*, 1996; MacKenzie *et al.*, 1994b, 1995; Ong *et al.*, 1999; Smith *et al.*, 1988; Yamamoto *et al.*, 2000). These include outbreaks associated with both drinking water and recreational water (swimming pools and water parks). With the adoption of more stringent treatments of source water by the water industry after the massive cryptosporidiosis outbreak in Milwaukee in 1993, the number of drinking water-associated outbreaks is in decline in the United States and United Kingdom in recent years. Even though five *Cryptosporidium* spp. are commonly found in humans, thus far only *C. parvum* and *C. hominis* are associated with cryptosporidiosis outbreaks, with *C. hominis* responsible for more outbreaks than *C. parvum* (McLauchlin *et al.*, 2000; Peng *et al.*, 1997; Xiao *et al.*, 2003). This is even the case for the United Kingdom, where *C. parvum* is more common than *C. hominis* in the general population. In outbreak settings, immunocompetent adults may have voluminous but self-limiting diarrhea, with or without abdominal cramps, fatigue, vomiting, fever, and other symptoms (MacKenzie *et al.*, 1994a; Yamamoto *et al.*, 2000). Attack rates and incidence of specific clinical symptoms (diarrhea, vomiting, abdominal cramps, headache, fever, etc.) differ among outbreaks, though the reason for these variations is not known (Quiroz *et al.*, 2000).

Surveys conducted in various regions of the United States have demonstrated the presence of *Cryptosporidium* oocysts in 67–100% wastewaters, 24–100% of

surface waters, and 3.8–40% drinking waters (LeChevallier *et al.*, 1991a, 1991b; Madore *et al.*, 1987; Rose, 1997). The identity and human infective potential of these waterborne oocysts are not known, although it is likely that not all oocysts are from human-infecting *Cryptosporidium* species. Likewise, the source of the oocyst contamination is also not fully clear. Farm animals and human sewage discharge are generally considered to be major sources of surface water contamination with *C. parvum* (Meinhardt *et al.*, 1996). Because *Cryptosporidium* infection is common in wildlife, it is conceivable that wildlife can also be a source for *Cryptosporidium* oocysts in waters (Rose, 1997). The source for contamination (i.e., with oocysts of human or animal origin) involved in individual outbreaks, however, is frequently not known, largely due to the lack of investigations using suitable strain-specific diagnostic tools.

4.4.5 Foodborne Transmission

The role of food in the transmission of cryptosporidiosis is much less clear. Cryptosporidium oocysts have been isolated from several foodstuffs and these have mainly been associated with fruits, vegetables, and shellfish (Table 4.2). A survey of produce sold in Lima, Peru where *Cryptosporidium* is prevalent in humans demonstrated that 14.5% of samples were *Cryptosporidium* positive. In Norway, where sporadic *Cryptosporidium* infection rates are presumably lower, *Cryptosporidium* oocysts were found in 4% of fresh produce (Robertson and Gjerde, 2001b). Oysters, clams, mussels, and cockles in many countries have been shown to be contaminated with *Cryptosporidium* oocysts (Table 4.2). The association of oocyst contamination with these produce is particularly important from a public health viewpoint, as these products are frequently consumed raw without any thermal processing to inactivate oocysts. Mollusc filter feeders such as oysters, mussels, and clams pose a risk because they can concentrate pathogens from large volumes of potentially contaminated water, and *Cryptosporidium* oocysts found in them are frequently viable for extended periods of time (Fayer *et al*., 1998, 1999, 2002; Freire-Santos *et al*., 2001; Gomez-Bautista *et al*., 2000; Gomez-Couso *et al*., 2003a, 2003b; Tamburrini and Pozio, 1999).

Direct contamination of food by fecal materials from animals or food-handlers has been implicated in several foodborne outbreaks of cryptosporidiosis in industrialized nations (Millard *et al.*, 1994; Quiroz *et al.*, 2000). This is also likely a major source of contamination of fresh produce in endemic areas. Because *Cryptosporidium* oocysts are commonly found in surface water, contamination of fresh produce through irrigation or washing is probably also common (Armon *et al.*, 2002; Robertson and Gjerde, 2001b; Thurston-Enriquez *et al.*, 2002). In addition, marine water may also be contaminated with *Cryptosporidium* oocysts due to sewage discharge and agricultural runoff, which can in turn contaminate shellfish (Fayer *et al.*, 1998; Graczyk *et al.*, 2000). Studies conducted in various countries have found *C. parvum*, *C. hominis*, and *C. meleagridis* in shellfish, but in most areas, *C. parvum* is responsible for more than 80% of the contamination (Table 4.2), indicating agricultural runoff is probably the most important source for *Cryptosporidium* contamination in shellfish.

Table 4.2. Prevalence of *Cryptosporidium* in raw fruits, vegetables, and shellfish.

Food type	*Country*	*Prevalence*	*Species*	*Reference*
		Vegetables		
Vegetables	Costa Rica	Cilantro leaves: 4/80; Cilantro roots: 7/80; Lettuce: 2/80; Radish: 1/80; Carrot: 1/80; Tomato: 1/80; Cucumber: 1/80; Cabbage: 0/80		(Monge and Arias, 1996; Monge *et al.*, 1996)
Vegetables	Peru	Vegetables (cabbage, celery, cilantro, green onion, ground green chili, Leek, lettuce, parsley, yerba Buena, huacatay): 28/172		(Ortega *et al.*, 1997)
Fruits and vegetables	Norway	Alfalfa: 0/16; Dill: 0/7; Lettuce: 5/125 Mung bean sprouts: 14/149; Mushrooms: 0/55 Parsley: 0/7 Precut salad: 0/38 Radish sprouts: 0/6; Raspberries: 0/10; Strawberries: 0/62		(Robertson and Gjerde, 2001b; Robertson *et al.*, 2002b)
Sprout	Norway			
		Shellfish		
Clams	Spain and Italy	*Dosinia exoleta, Ruditapes philippinarum, Venerupis pullastra, Venerupis rhomboideus, Venus verrucosa:*1 0/17		(Freire-Santos *et al.*, 2000)
	Spain	*Dosinia exoleta, Venerupis pullastra, Venerupis rhomboideus, Venus verrocosa*: 10/18	*C. parvum* and *C. hominis*	(Gomez-Couso *et al.*, 2004)
	Spain and EU countries	*Dosinia exoleta, Venerupis pullastra, Venerupis rhomboideus, Venus verrocosa*: 20/68		(Gomez-Couso *et al.*, 2003a)
	Italy	*Chamelea gallina*: 2 of 16 pooled clams (30 clams/pool)	*C. parvum*	(Traversa *et al.*, 2004)
	Eastern USA and Canada	Clams: 3/375 (0.8)		(Fayer *et al.*, 2003)

(*continued*)

Table 4.2. (*continued*)

Food type	*Country*	*Prevalence*	*Species*	*Reference*
Cockles	Spain	*Cerastoderma edule*: postive/6	*C. parvum*	(Gomez-Bautista *et al.*, 2000)
	Spain and EU countries	*Cerastoderma edule*: 5/24		(Gomez-Couso *et al.*, 2003a)
Mussels	Spain	*Mytilus galloprovincialis*: positive/180	*C. parvum*	(Gomez-Bautista *et al.*, 2000)
	Spain	*Mytilus galloprovincialis*: 12/22	*C. parvum*	(Gomez-Couso *et al.*, 2004)
	Spain	*Mytilus galloprovincialis*: 6/15		(Freire-Santos *et al.*, 2000)
	Spain and EU countries	*Mytilus galloprovincialis*: 35/107		(Gomez-Couso *et al.*, 2003a)
	Northern Ireland	*Mytilus edulis*: 2/16	*C. hominis*	(Lowery *et al.*, 2001b)
	Canada	Zebra mussel (*Dreissena ploymorpha* 32/32 pools (514 mussels total)	*C. hominis*	(Graczyk *et al.*, 2001)
	USA	Bent mussel (*Ischadium recurvum*): 14/16		(Graczyk *et al.*, 1999)
	Ireland	*Mytilus edulis*: 3/26 pools (10 mussels/pool)		(Chalmers *et al.*, 1997)
Oysters	Chesapeake Bay, USA	*Crassostrea virginica*: 142/360	*C. parvum* and *C. hominis*	(Fayer *et al.*, 1998)
	Chesapeake Bay, USA	Commercial *Crassostrea virginica*: 182/510	*C. parvum* and *C. hominis*	(Fayer *et al.*, 1999)
	Chesapeake Bay, USA	*Crassostrea virginica*: 331/1590	*C. parvum* and *C. hominis*	(Fayer *et al.*, 2002)
	Eastern USA and Canada	*Crassostrea virginica*: 32/550 (5.8%)	*C. parvum*, *C. hominis*, *C. meleagridis*	(Fayer *et al.*, 2003)
	Spain	*Ostrea edulis*: 5/6		(Freire-Santos *et al.*, 2000)
	Spain	*Ostrea edulis*: 6/9	*C. parvum* and *C. hominis*	(Gomez-Couso *et al.*, 2004)
	Spain and EU countries	*Ostrea edulis*: 23/42		(Gomez-Couso *et al.*, 2003a)

Very few case control studies have examined the role of potentially contaminated food as a risk factor in the acquisition of *Cryptosporidium* infection in endemic areas. A study conducted on children in Brazil failed to show any association between *Cryptosporidium* infection and diet or type of food hygiene (Pereira *et al.*, 2002a). Case control studies conducted in the United States, United Kingdom, and Australia have actually shown that eating raw vegetables has a protective role against *Cryptosporidium* infection in immunocompetent persons (Hunter *et al.*, 2004b; Robertson *et al.*, 2002a; Roy *et al.*, 2004). Nevertheless, foodborne outbreaks of cryptosporidiosis occurs frequently in the United States, United Kingdom, and other industrialized nations, usually due to consumption of contaminated fresh produce, apple cider, or milk (Anonymous, 1996, 1997, 1998; Gelletlie *et al.*, 1997; Millard *et al.*, 1994; Quiroz *et al.*, 2000). It is estimated that about 10% *Cryptosporidium* infections in the United States are foodborne (Mead *et al.*, 1999).

4.5 DETECTION AND DIAGNOSIS

4.5.1 Serologic Methods

Humans and animals infected with *Cryptosporidium* spp. develop antibodies against *Cryptosporidium* antigens (Mead *et al.*, 1988). Electrophoretic and Western blot analysis showed that specific antibody response appeared between day 4 and 15 post inoculation. The two main target antigens had apparent molecular weights of 15–17 and 23 kDa (Reperant *et al.*, 1994). These two antigens, Cp17 (also called gp15) and Cp23 (also called the 27 kDa antigen), have been used by many researchers in the detection of *Cryptosporidium* antibodies by enzyme-linked immunosorbent assays (ELISA) or Western blot (Caputo *et al.*, 1999; Frost *et al.*, 1998; Priest *et al.*, 1999, 2001). Usually, native Cp17 extracted by Triton from oocysts of *C. parvum* and recombinant Cp23 expressed in *E. coli* are used in these assays (Priest *et al.*, 1999; Wang *et al.*, 2003). ELISA methods using these two antigens generally have higher sensitivity and specificity than earlier methods (Leach *et al.*, 2000; Okhuysen *et al.*, 1998; Zu *et al.*, 1994) that use crude oocyst antigens (Priest *et al.*, 1999). Most researchers use both antigens in serologic studies. ELISA based on Cp17 and Cp23 have been used in many studies of *Cryptosporidium* transmission in immunocompromised persons (Eisenberg *et al.*, 2001), children (Steinberg *et al.*, 2004), the general community (Frost *et al.*, 2001, 2002, 2003, 2004), and in investigations of cryptosporidiosis outbreaks (McDonald *et al.*, 2001). Recently, a multiplex bead assay based on these two antigens has been developed for the detection of *Cryptosporidium* antibodies in sera and oral fluids (Moss *et al.*, 2004). These serologic assays are not intended for the diagnosis of active *Cryptosporidium* infection, as antibodies to both the 27- and 17-kDa antigens have a half-life of about 12 weeks (Priest *et al.*, 2001).

4.5.2 Methods for Detection of *Cryptosporidium* in Stool Specimens

At the moment, almost all active *Cryptosporidium* infections are diagnosed by analysis of stool specimens. Examination of intestinal or biliary biopsy is sometimes used in the diagnosis of cryptosporidiosis in AIDS patients (Clayton *et al.*, 1994).

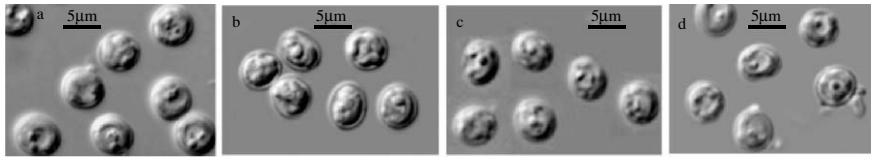

Figure 4.1. Oocysts of *Cryptosporidium parvum* (a) *C. hominis* (b) *C. meleagridis* (c) and *C. suis* (d) under differential interference contrast microscopy.

However, the sensitivity of the diagnosis depends on the location of tissues examined; duodenum is usually infected with *Cryptosporidium* only at high-intensity infection (Genta *et al.*, 1993), and the terminal ileum has significantly higher detection rates than the duodenum (Greenberg *et al.*, 1996). Thus, upper endoscopic biopsies are much less sensitive than lower endoscopic biopsies in diagnosing cryptosporidiosis. However, lower endoscopy is generally considered too invasive and risky for many AIDS patients.

Stool specimens are usually collected fresh or in fixative solutions such as 2.5% potassium dichromate or 10% buffered formalin (Garcia *et al.*, 1983), and are concentrated using either traditional ethyl acetate (Dubey, 1993) or Weber-modified ethyl-acetate concentration (Weber *et al.*, 1992). Sometimes other concentration methods such as sucrose, salt, or cesium chloride floatation are also used (Deng and Cliver, 1999b; Fayer *et al.*, 2000b; Kuczynska and Shelton, 1999; Kuhn *et al.*, 2002; Webster *et al.*, 1996), but they are mostly used in the analysis of fecal specimens from animals, which generally do not have as much lipids as human stool specimens. A variety of methods are used in the detection of *Cryptosporidium* in concentrated stool specimens, including microscopy, immunoassays, and molecular techniques (Arrowood, 1997). If clinical specimens will be analyzed by molecular methods, formalin should not be used as a fixative, as it would interfere with the analysis and reduce the efficiency of PCR amplification.

4.5.2.1 Microscopy

Concentrated stool specimens can be examined by microscopy in several ways. Frequently, when the number of oocysts is high, direct wet mount is made and *Cryptosporidium* oocysts are detected by bright-field microscopy. This allows the observation of oocysts morphology and more accurate measurement of oocysts, which is frequently needed in biologic studies. More often, differential interference contrast (DIC) is used in microscopy, which produces better images and visualization of internal structures of oocysts (Fig. 4.1). Morphology and morphometrics measurements, however, are generally not enough for *Cryptosporidium* species differentiation (Fall *et al.*, 2003; Xiao *et al.*, 2004a), as many species of *Cryptosporidium* look similar under microscopes and have similar morphometrics measurements (Table 4.3, Fig. 4.1). In general, oocysts of gastric *Cryptosporidium* species are bigger and more ovoid and those of intestinal species are smaller and more spherical (Table 4.3).

More often, *Cryptosporidium* oocysts in concentrated stool specimens are detected by microscopy after staining of the fecal smears. Many special stains have

Table 4.3. Morphometric measurements of established *Cryptosporidium* species[a].

Species	*No. of oocysts measured*	*Length in μm (mean)*	*Width in μm (mean)*	*Length/width (mean)*	*Reference*
Gastric					
C. andersoni	50	6.0–8.1 (7.4)	5.0–6.5 (5.5)	1.07–1.50 (1.35)	(Lindsay *et al.*, 2000)
C. galli	50	8.0–8.5 (8.25)	6.2–6.4 (6.30)	1.30	(Ryan *et al.*, 2003)
C. molnari[b]	22	3.23–5.45 (4.72)	3.02–5.04 (4.47)	1.00–1.17 (1.05)	(Alvarez-Pellitero and Sitja-Bobadilla, 2002)
C. muris	25	8.0–9.0 (8.4)	5.6–6.4 (6.1)	1.25–1.61 (1.38)	(Palmer *et al.*, 2003)
C. serpentis	37	5.82–6.06 (5.94)	4.35–5.19 (5.11)	1.14–1.20 (1.17)	(Xiao *et al.*, 2004c)
Intestinal					
C. baileyi[c]	25	5.6–6.3 (6.2)	4.5–4.8 (4.6)	1.2–1.4 (1.4)	(Current *et al.*, 1986)
C. bovis	50	4.76–5.35 (4.89)	4.17–4.76 (4.63)	1.06	(Fayer *et al.*, 2005)
C. canis	200	3.68–5.88 (4.95)	3.68–5.88 (4.71)	1.04–1.06 (1.05)	(Fayer *et al.*, 2001)
C. felis	40	3.2–5.1 (4.6)	3.0–4.0 (4.0)	1.15	(Sargent *et al.*, 1998)
C. hominis	100	4.4–5.9 (5.20)	4.4–5.4 (4.86)	1.00–1.09 (1.07)	(Morgan-Ryan *et al.*, 2002)
C. meleagridis	55	4.93 (CL = 0.06)[d]	4.40 (CL = 0.05)[d]	1.12 (CL = 0.02)[d]	(Xiao *et al.*, 2004a)
C. parvum	100	4.70–6.00 (5.19)	4.41–5.95 (4.90)	1.05–1.06 (1.06)	(Fayer *et al.*, 2001)
C. saurophilum	20	4.81–5.07 (4.94)	4.35–4.63 (4.49)	1.11–1.17 (1.14)	(Xiao *et al.*, 2004c)
C. suis	50	4.4–4.9 (4.6)	4.0–4.3 (4.2)	1.1	(Ryan *et al.*, 2004b)
C. wrairi	30	4.8–5.6 (5.4)	4.0–5.0 (4.6)	1.04–1.33 (1.17)	(Tilley *et al.*, 1991)

[a] Whenever possible, measurements from parasites confirmed by molecular or biologic characterizations are quoted.
[b] Also found in the intestine.
[c] Also found in the respiratory tract.
[d] CL: 95% confidence limit.

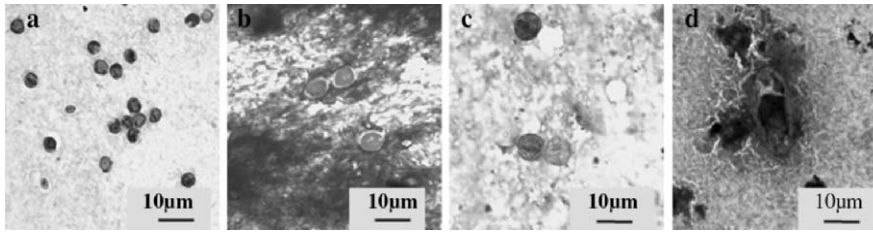

Figure 4.2. Acid-fast stained oocysts of *Cryptosporidium hominis* (a), *C. muris* (b), *Isospora belli* (c), and *Cyclospora cayetanensis* (d) under bright-field microscopy.

been used in the detection of *Cryptosporidium* oocysts, but acid-fast stains are the most often used (Arrowood, 1997). Modified acid-fast staining is very commonly used in developing countries because of its low cost, easy use, no need for special microscopes, and simultaneous detection of several other pathogens such as *Isospora* and *Cyclospora* (Fig. 4.2). Two acid-fast staining widely used in *Cryptosporidium* oocyst detection are the modified Ziehl-Neelsen acid-fast staining and modified Kinyoun's acid-fast staining (Arrowood, 1997).

Recently, immunofluorescence assays (IFA) have been used increasingly in *Cryptosporidium* oocyst detection by microscopy, especially in industrialized nations. Compared to acid-fast staining, IFA has higher sensitivity and specificity (Arrowood and Sterling, 1989; Johnston *et al.*, 2003; Quilez *et al.*, 1996). Many commercial IFA kits are marketed for the diagnosis of *Cryptosporidium*, some of which include reagents allowing simultaneous detection of *Giardia* cysts (Fig. 4.3). These include Merifluor *Cryptosporidium*/*Giardia* kit from Meridian Bioscience,

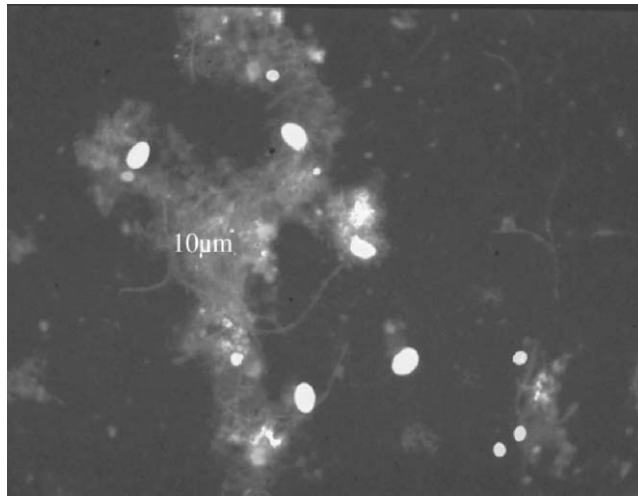

Figure 4.3. *Cryptosporidium parvum* oocysts (small apple green objects) and *Giardia duodenalis* cysts (large apple green objects) under immunofluorescence microscopy.

Giardia/Crypto IF kit from TechLab, Monofluo *Cryptosporidium* kit from Sanofi Diagnostics Pasteur, Crypto/*Giardia* Cel kit from TCS Biosciences, and Aqua-Glo G/C kit from Waterborne, etc. Because of the high sensitivity and specificity, IFA has been used in some studies as the gold standard or as a reference test (Garcia and Shimizu, 1997; Johnston *et al.*, 2003). It has been shown that most antibodies used in immunofluorescence detection of *Cryptosporidium* oocysts recognize carbohydrate epitopes on the oocyst wall (Moore *et al.*, 1998; Yu *et al.*, 2002). As the monoclonal antibodies used in commercial IFA kits react with oocysts of almost all *Cryptosporidium* species, IFA cannot make diagnosis at the species level (Graczyk *et al.*, 1996; Yu *et al.*, 2002).

The sensitivity of most microscopic methods is probably low. The detection limit for the combination of ethyl acetate concentration and IFA was shown to be 10,000 oocysts per gram of liquid stool and 50,000 oocysts per gram of formed stool (Weber *et al.*, 1991; Webster *et al.*, 1996). The sensitivity of acid-fast staining was 10-fold lower (Weber *et al.*, 1991), probably because acid-fast stains do not always consistently stain all oocysts (Garcia *et al.*, 1987). Replacing the ethyl acetate concentration procedure with sucrose, cesium chloride, or sodium chloride floatation can increase the sensitivity to 30–200 oocysts per gram of feces in animal studies (Deng and Cliver, 1999b; Fayer *et al.*, 2000b; Kuczynska and Shelton, 1999). These concentration techniques, however, are rarely used in diagnostic analysis of human stool specimens. For now, it is recommended that whenever possible, multiple specimens from each patient should be examined in the diagnosis of *Cryptosporidium* infection, as carriers with low oocyst shedding are common (Roberts *et al.*, 1989), and examination of individual specimens can lead to the detection of only 53% of infections (Greenberg *et al.*, 1996).

4.5.2.2 Antigen Detection by Immunoassays

Cryptosporidium infection can also be diagnosed by the detection of *Cryptosporidium* antigens in stool specimens by immunoassays. Antigen-capture-based enzyme immunoassays (EIA) have been used in the diagnosis of cryptosporidiosis since 1990 (Anusz *et al.*, 1990; Chapman *et al.*, 1990; Garcia and Shimizu, 1997; Rosenblatt and Sloan, 1993; Ungar, 1990). In recent years, they have gained popularity because of the ability to screen a large number of samples and an experienced microscopists is not required. Several commercial EIA kits are commonly used, such as the Alexon-Trend ProSpecT *Cryptosporidium* Microplate Assay and Meridian Premier *Cryptosporidium* kit. High specificity (99–100%) has been generally reported for these EIA kits (Dagan *et al.*, 1995; Garcia and Shimizu, 1997; Johnston *et al.*, 2003; Parisi and Tierno, 1995; Siddons *et al.*, 1992). Various sensitivities, however, have been reported, ranging from 70 (Johnston *et al.*, 2003) to 94–100% (Bialek *et al.*, 2002; Dagan *et al.*, 1995; Garcia and Shimizu, 1997; Parisi and Tierno, 1995; Rosenblatt and Sloan, 1993; Siddons *et al.*, 1992). Nevertheless, occasional false-positivity of EIA kits is known to occur in the detection of *Cryptosporidium* (Chapman *et al.*, 1990), and at least one manufacturer's recall of EIA kits has occurred because of high nonspecificity (Anonymous, 1999). These kits generally do not perform well when the number of oocysts in specimens is small (Ignatius *et al.*, 1997; Johnston *et al.*, 2003). Almost all EIA kits are for the detection of only *Cryptosporidium*,

but a triage parasite panel EIA has also been marketed for simultaneous detection of *Giardia duodenalis*, *Entamoeba histolytica/E. dispar*, and *Cryptosporidium* antigens in human stool specimens (Garcia *et al.*, 2000). Most of the EIA kits have been evaluated only with human stool specimens. Their usefulness in the detection of *Cryptosporidium* spp. in animals may be compromised by the high specificity of antibodies. For example, the ProSpecT *Cryptosporidium* EIA does not detect many *Cryptosporidium* species that are genetically distant from *C. parvum*, such as *C. muris*, *C. andersoni*, *Cryptosporidium Serpentis*, and *Cryptosporidium baileyi* (Graczyk *et al.*, 1996).

In the last few years, at least four lateral flow immunochromatographic assays have been marketed for rapid detection of *Cryptosporidium* in stool specimens: the ImmunoCard STAT! *Cryptosporidium*/*Giardia* rapid assay (Meridian Bioscience), ColorPAC *Cryptosporidium*/*Giardia* rapid assay (Becton Dickinson), RIDA Quick *Cryptosporidium*/*Giardia* Combi (R-Biopharm), and the *Cryptosporidium* Dipstick (Cypress Diagnostics) (Garcia and Shimizu, 2000; Garcia *et al.*, 2003; Johnston *et al.*, 2003). In a few evaluation studies conducted with two of the assays, they have been shown to have high specificities (> 98%) (Garcia and Shimizu, 2000; Garcia *et al.*, 2003; Johnston *et al.*, 2003; Katanik *et al.*, 2001). The sensitivities of these assays were also high (98–100%) in earlier studies (Garcia and Shimizu, 2000; Garcia *et al.*, 2003; Katanik *et al.*, 2001). However, a recent study has shown a sensitivity of 68% for one of the assays (Johnston *et al.*, 2003). These rapid assays have also been plagued by quality problems and have been subjected to several manufacturer's recalls because of false positivity (Anonymous, 2002, 2004).

4.5.2.3 Molecular Methods

Molecular techniques, especially PCR and PCR-related methods, have been developed and used in the detection and differentiation of *Cryptosporidium* spp. for many years. Earlier PCR methods (Chrisp and LeGendre, 1994; Johnson *et al.*, 1995; Laxer *et al.*, 1991; Webster *et al.*, 1993) do not have the ability for species-differentiation or genotyping, and can thus only be used in the determination of the presence or absence of *Cryptosporidium* spp. The primer sequences of these techniques, with the exception of those by Johnson *et al.* (1995), are mostly based on undefined genomic sequences from *C. parvum* bovine isolates. These sequences tend to be more polymorphic than structural and house-keeping genes, therefore the primers based on them are unlikely to efficiently amplify DNA from *Cryptosporidium* spp. (such as *C. muris*, *C. baileyi*, *C. serpentis*, *C. canis*, and *C. felis*) and genotypes (such as the fox, skunk, and opossum genotypes) that are more distant from *C. parvum*.

Several PCR-RFLP based genotyping tools have been developed for the detection and differentiation of *Cryptosporidium* at the species level (Amar *et al.*, 2004; Awad-el-Kariem *et al.*, 1994; Kimbell *et al.*, 1999; Leng *et al.*, 1996; Lowery *et al.*, 2000; Nichols *et al.*, 2003; Sturbaum *et al.*, 2001; Xiao *et al.*, 1999a, 1999b). Most of these techniques are based on the SSU rRNA gene. However, one of the method uses an array of primers (23 primers in a nested PCR) to cover all combinations of sequence heterogeneity in the primer region of the COWP gene (Amar *et al.*, 2004). Unfortunately, primers of some of the SSU rRNA-based techniques (Awad-el-Kariem *et al.*, 1994; Kimbell *et al.*, 1999; Leng *et al.*, 1996) used conserved

sequences of eukaryotic organisms. Therefore, these primers also amplify DNA from organisms other than *Cryptosporidium* (Sulaiman *et al.*, 1999). The technique by Sturbaum *et al.* (2001) also amplifies DNA of dinoflagellates (Sturbaum *et al.*, 2002). A PCR-RFLP analysis of the internal transcribed spacers of the rRNA gene can also differentiate *C. felis* from *C. parvum* (Morgan *et al.*, 1999a). Nucleotide sequencing-based approaches have also been developed for the differentiation of various *Cryptosporidium* spp. (Morgan *et al.*, 1998, 1999a; Sulaiman *et al.*, 2000, 2002; Ward *et al.*, 2002). Not all these molecular techniques, however, are diagnostic methods by nature because some of them use long amplicons (Sulaiman *et al.*, 2000, 2002), and some also amplify other apicomplexan parasites and dinoflagellates (Ward *et al.*, 2002).

Other genotyping techniques are mostly for the differentiation of *C. parvum* and *C. hominis* (Bonnin *et al.*, 1996; Carraway *et al.*, 1996, 1997; Morgan *et al.*, 1995, 1996, 1997; Patel *et al.*, 1998, 1999; Peng *et al.*, 1997; Rochelle *et al.*, 1999; Spano *et al.*, 1997, 1998; Sulaiman *et al.*, 1998; Widmer, 1998;). Both parasites have been identified in humans, but *C. hominis* (the anthroponotic genotype) has been almost exclusively found in humans; whereas the *C. parvum* (the zoonotic genotype) infects humans, ruminants, and a few other animals. Many of the genotyping tools used in these studies, however, cannot detect and differentiate other *Cryptosporidium* spp. or genotypes. Their usefulness in the analysis of human stool specimens is compromised by the failure to detect *C. canis* and *C. felis*. Indeed, a recent study has compared the ability of 10 commonly used genotyping tools in detecting seven human-pathogenic *Cryptosporidium* species/genotypes. With the exception of SSU rRNA-based PCR tools, which detected all seven *Cryptosporidium* species/genotypes, most of the genotyping tools examined had only the ability to detect *C. parvum*, *C. hominis*, and *C. meleagridis* (Jiang and Xiao, 2003).

Several subtyping tools have also been developed to characterize the diversity within the *C. parvum* or *C. hominis*. One of the most commonly used techniques is microsatellite analysis. Even though initial characterizations of eight microsatellite loci had identified only limited intragenotypic genetic diversity in *C. parvum* and *C. hominis* (Aiello *et al.*, 1999), more recent studies have identified several microsatellite sequences that seem to be more variable (Alves *et al.*, 2003a; Caccio *et al.*, 2000, 2001; Feng *et al.*, 2000; Mallon *et al.*, 2003a, 2003b; Widmer *et al.*, 2004). Although not a strict microsatellite locus by definition, results of a series of recent studies have shown high sequence polymorphism in the gene of 60 kDa glycoprotein precursor (GP60; also known as gp15/45/60, gp40/15) (Leav *et al.*, 2002; Peng *et al.*, 2001, 2003a, 2003b; Strong *et al.*, 2000; Sturbaum *et al.*, 2003; Sulaiman *et al.*, 2001; Wu *et al.*, 2003; Zhou *et al.*, 2003). Most of the genetic heterogeneity in the gene is present in the number of a tri-nucleotide repeat (TCA, TCG, or TCT), although extensive sequence differences are also present between groups (allele families) of subtypes. Other subtyping tools include sequence analysis of HSP70 (Peng *et al.*, 2003a; Sulaiman *et al.*, 2001), heteroduplex analysis and nucleotide sequencing of the double-stranded RNA (Leoni *et al.*, 2003; Xiao *et al.*, 2001b), and single-strand conformation polymorphism (SSCP)-based analysis of the second internal transcribed spacer (ITS-2) (Gasser *et al.*, 2003, 2004). A multilocus

mini- and micro-satellite subtyping tool for *C. parvum* and *C. hominis* have also been developed (Mallon *et al.*, 2003a, 2003b). The usefulness of subtyping tools has been demonstrated by the analysis of samples from foodborne and waterborne outbreaks of cryptosporidiosis (Glaberman *et al.*, 2002; Leoni *et al.*, 2003; Sulaiman *et al.*, 2001; Xiao *et al.*, 2001b, 2003).

A few PCR related techniques have also been used in the quantitation and viability evaluation of *Cryptosporidium* oocysts. An excystation procedure prior to DNA extraction and PCR (excystation-PCR) has been developed to detect viable *C. parvum* oocysts (Filkorn *et al.*, 1994; Wagner-Wiening and Kimmig, 1995). Similarly, others have used a combination of cell culture and PCR (Di Giovanni *et al.*, 1999; Rochelle *et al.*, 1996; LeChevallier *et al.*, 2003) or RT-PCR (Rochelle *et al.*, 1997b) (CC-PCR or CC-RT-PCR) to detect viable *Cryptosporidium* oocysts. Because in theory RNA is less stable than DNA and breaks down quickly by the released RNAse during cell death, several reverse transcription-PCR (RT-PCR) techniques have been described for the detection of viable oocysts (Hallier-Soulier and Guillot, 2003; Jenkins *et al.*, 2000; Kaucner and Stinear, 1998; Stinear *et al.*, 1996; Widmer *et al.*, 1999). However, RNA breakdown is a slow process, which may lead to an overestimate of the viability of oocysts (Fontaine and Guillot, 2003). By nature, most of the techniques do not differentiate *Cryptosporidium* species or genotypes, although one research group used sequence analysis to determine genotypes (Di Giovanni *et al.*, 1999; LeChevallier *et al.*, 2003). More recently, several real-time PCR methods have been developed, which allow quick detection and even quantification of *Cryptosporidium* oocysts (Fontaine and Guillot, 2002, 2003; Higgins *et al.*, 2001; Limor *et al.*, 2002; MacDonald *et al.*, 2002; Tanriverdi *et al.*, 2002;). One of the techniques can differentiate *C. parvum* from *C. hominis* (Tanriverdi *et al.*, 2002), whereas another can differentiate the five common *Cryptosporidium* species in humans (Limor *et al.*, 2002). A new integrated detection assay combining capture of double-stranded RNA with probe-coated beads, RT-PCR, and lateral flow chromatography has also been developed, which should also shorten detection time (Kozwich *et al.*, 2000).

Molecular tools other than PCR have also been developed for the detection and/or differentiation of *Cryptosporidium*. Fluorescence *in situ* hybridization (FISH) or colorimetric *in situ* hybridization of probes to the SSU rRNA has been used in the detection or viability evaluation of *C. parvum* oocysts (Lindquist *et al.*, 2001b; Rochelle *et al.*, 2001; Smith *et al.*, 2004; Vesey *et al.*, 1998). It probably does not have higher sensitivity than microscopy, but with further development, it may be used in the differentiation of the species/genotypes of *Cryptosporidium* oocysts on microscope slides. Nucleic acid sequence-based amplification (NASBA) has been used in the detection of viable *C. parvum* oocysts (Baeumner *et al.*, 2001). More recently, a biosensor technique for the detection of viable *C. parvum* oocysts has also been described (Baeumner *et al.*, 2004), and a microarray technique based on HSP70 sequence polymorphism has been developed to differentiate *Cryptosporidium* genotypes (Straub *et al.*, 2002).

The following are some of the examples of the usages of molecular tools in epidemiologic investigations of human *Cryptosporidium* infections (Xiao and Ryan, 2004; Xiao *et al.*, 2003).

(A) Establishment of the identity of *Cryptosporidium* spp. in humans. We can now identify the species of *Cryptosporidium* that infects humans, the potential for non-*C. parvum Cryptosporidium* spp. to infect humans, the proportion of infections attributable to each species in various socioeconomic and epidemiologic settings, and the heterogeneity within each species causing human infections (Pedraza-Diaz *et al.*, 2000; Pieniazek *et al.*, 1999; Xiao *et al.*, 2001a).

(B) Identification of infection or contamination sources. When used in conjunction with traditional epidemiologic investigations, molecular tools can help identify the source of infection or contamination: Anthroponotic versus zoonotic *Cryptosporidium* infection, farm animal or companion animal origin versus wildlife origin. With a large sample size, molecular tools can help assess the human infective potential of *Cryptosporidium* spp. from various animals that are in frequent contact with humans. With higher resolution tools, molecular techniques can make a direct linkage between human cases of cryptosporidiosis and contamination sources (contaminated food item or water source, human index case, e.g., a foodhandler, animal reservoir) (Alves *et al.*, 2003b; Glaberman *et al.*, 2002; Hunter *et al.*, 2003; Learmonth *et al.*, 2004; McLauchlin *et al.*, 2000).

(C) Characterization of transmission dynamics of cryptosporidiosis in communities. High-resolution molecular tools can help to distinguish cryptosporidiosis point-source outbreaks from endemic but unrelated clusters of cases. These tools may also serve to identify common transmission pathways, distinguish multiple episodes of infections in humans, elucidate mechanisms of immunity against homologous and heterologous *Cryptosporidium* spp., and differentiate new episodes of infection from reactivation of latent infection (Alves *et al.*, 2003b; Cama *et al.*, 2003; Hunter *et al.*, 2004b; Peng *et al.*, 2003a; Xiao *et al.*, 2001a).

(D) Characterization of clinical spectrum and pathobiology of cryptosporidiosis. Molecular tools can improve understanding of the mechanisms underlying the variable clinical presentations and attack rates in outbreaks, variations in disease spectrum in AIDS patients, and differences in infection sites and pathophysiology caused by *Cryptosporidium* spp. In addition to host susceptibility, it is likely that the genetic diversity of *Cryptosporidium* spp. plays an important role in the clinical and pathologic spectrum of human cryptosporidiosis (Hashim *et al.*, 2004; Hunter *et al.*, 2004a; McLauchlin *et al.*, 1999; Pereira *et al.*, 2002b; Xiao *et al.*, 2001a).

4.5.3 Methods for Detection of *Cryptosporidium* Oocysts in Environmental Samples

4.5.3.1 Detection of *Cryptosporidium* Oocysts in Water Samples

Currently, the identification of *Cryptosporidium* oocysts in environmental samples is largely made by the use of IFA after concentration processes (EPA ICR method, EPA method1622/1623, United Kingdom SCA method, and United Kingdom regulatory method) (Lindquist *et al.*, 2001a). This generally requires the filtration of 10–100 L or more water, concentration and isolation of oocysts, staining of oocysts with FITC-labeled *Cryptosporidium* antibodies, and examination and quantitation of oocysts by microscopy. In the ICR or SCA method, nominal 1 μm 10" cartridge filters are used for filtration and floatation (using Percoll, sucrose or tripotassium citrate) is used

in oocyst concentration. In method 1622/1622 and the United Kingdom regulatory method, capsule filters are used in filtration and immounomagnetic separation is used in oocyst concentration. In addition, 4', 6- diamidoino-2-phenylindole (DAPI) vital dye is used in these newer methods for counterstaining. As a result, the sensitivity and accuracy of the newer methods have been improved. The recovery rates of the EPA method 1622/1623 for *Cryptosporidium* oocysts have been reported to be between 10 and 75% for surface water (DiGiorgio *et al.*, 2002; Hsu, 2003; LeChevallier *et al.*, 2003; Simmons *et al.*, 2001; Ware *et al.*, 2003). The EPA methods 1622 and 1623 can be downloaded at http://www.epa.gov/nerlcwww/1622ap01.pdf and http://www.epa.gov/waterscience/methods/1623.pdf, respectively. The United Kingdom regulatory method can be downloaded at http://www.dwi.gov.uk/regs/crypto/pdf/sop%20part%202.pdf. It should be noted that cross-reactivity of the monoclonal antibodies used in the IMS and IFA kits has been reported with dinoflagellates (Sturbaum *et al.*, 2002) and algae (Rodgers *et al.*, 1995), which may interfere with accurate detection and quantitation of *Cryptosporidium* oocysts in water, and requires careful examinations of oocyst internal structure by DAPI staining and DIC microscopy.

Because IFA detects oocysts from all *Cryptosporidium* spp., the species distribution of *Cryptosporidium* oocysts in environmental samples cannot be assessed. Although many surface water samples contain *Cryptosporidium* oocysts, it is unlikely that all of these oocysts are from human-pathogenic species or genotypes, because only five *Cryptosporidium* spp. (*C. parvum*, *C. hominis*, *C. meleagridis*, *C. canis*, and *C. felis*) are responsible for most human *Cryptosporidium* infections. Information on the source of *Cryptosporidium* contamination is necessary for accurate risk assessment, effective evaluation, and selection of management practices for reducing *Cryptosporidium* contamination in surface water and the risk of cryptosporidiosis. Thus, identification of oocysts to the species/genotype level is of significant public health importance.

The performance of many PCR methods in the analysis of environmental samples have been evaluated with *Cryptosporidium* negative samples seeded with known numbers of *C. parvum* oocysts. In early studies, PCR or RT-PCT was performed on DNA extracted directly from water concentrates seeded with *Cryptosporidium* oocysts with no oocyst isolation procedures or mere Percoll-sucrose floatation (Chung *et al.*, 1998, 1999; Kaucner and Stinear, 1998; Mayer and Palmer, 1996; Monis and Saint, 2001; Rochelle *et al.*, 1997a, 1997b; Sluter *et al.*, 1997; Stinear *et al.*, 1996). Variable sensitivities were reported by these studies, ranging from 1 to more than 100 oocysts per sample. Many researchers observed an inhibitory effect of surface water on PCR (Chung *et al.*, 1998; Johnson *et al.*, 1995; Lowery *et al.*, 2000; Rochelle *et al.*, 1997a; Sluter *et al.*, 1997; Xiao *et al.*, 2000a). Thus, almost all recent techniques have used an IMS procedure prior to cell culture and/or DNA extraction to remove PCR inhibitors or contaminants present in water samples (Di Giovanni *et al.*, 1999; Hallier-Soulier and Guillot, 1999, 2000, 2003; Johnson *et al.*, 1995; Jellison *et al.*, 2002; Kostrzynska *et al.*, 1999; Lowery *et al.*, 2000, 2001a, 2001b; Nichols *et al.*, 2003; Rimhanen-Finne *et al.*, 2002; Sturbaum *et al.*, 2002; Ward *et al.*, 2002; Wu *et al.*, 2000; Xiao *et al.*, 2000a, 2001c).

The presence of host-adapted *Cryptosporidium* species and genotypes make it possible to develop genotyping tools to determine whether the *Cryptosporidium* oocysts found in waters are from human-infective species, and to track the source of *Cryptosporidium* oocyst contamination in water. One of such techniques, the SSU rRNA-based nested PCR-RFLP method, has been successfully used in conjunction with IMS in the detection and differentiation of *Cryptosporidium* oocysts present in storm water, raw surface water, and wastewater (Xiao *et al.*, 2000a, 2001c, 2004b). In one study, 29 water samples were collected after storms, from a stream that contributes to the New York City Water Supply system and analyzed. They showed the presence of 12 wildlife genotypes of *Cryptosporidium* in 27 samples. Twelve of the 27 PCR positive samples had multiple genotypes. Four of the genotypes were traced to sources (*C. baileyi* from birds, an unnamed species from snakes, and 2 genotypes from opossums), whereas the rest were presumed to be wildlife genotypes that have never been found in humans or domestic animals, suggesting that wildlife was a major contributor for *Cryptosporidium* oocyst contamination in storm water (runoffs) in the area studied. This finding was consistent with the environmental setting (catchments were forested and isolated from agricultural activities) of the sampling site (Xiao *et al.*, 2000a).

The same technique was used in the analysis of raw surface water samples collected from different locations (Maryland, Wisconsin, Illinois, Texas, Missouri, Kansas, Michigan, Virginia, and Iowa) in the United States. A total of 55 samples were analyzed, 25 of which produced positive PCR amplification. Only 4 *Cryptosporidium* genotypes (*C. parvum*, *C. hominis*, *C. andersoni*, and *C. baileyi*) were found, all of which are parasites commonly found in farm animals and/or humans, indicating that humans and farm animals are major sources of *Cryptosporidium* oocyst contamination in these waters. Similar results were also obtained from 49 raw wastewater samples (10 or 50 ml of grab samples) collected from a treatment plant in Milwaukee, WI, 12 of which were positive for *Cryptosporidium*. Seven *Cryptosporidium* spp. (*C. parvum*, *C. hominis*, *C. andersoni*, *C. muris*, *C. canis*, *C. felis*, and *Cryptosporidium* cervine genotype) were found, with *C. andersoni* as the most common *Cryptosporidium*. As expected, the diversity of *Cryptosporidium* spp. found in source and wastewaters was much lower than that in storm waters (Xiao *et al.*, 2001c).

Two SSU rRNA-based PCR-sequencing tools and one other SSU-based PCR-RFLP tool have also been used successfully in the differentiation of *Cryptosporidium* oocysts in surface and wastewater samples (Jellison *et al.*, 2002; Nichols *et al.*, 2003; Ward *et al.*, 2002). Sequences of *C. muris*, *C. andersoni*, and presumed *C. baileyi* were obtained from seven samples of surface water from a watershed in Massachusetts (Jellison *et al.*, 2002). Analysis of 17 positive surface water samples and 6 wastewater samples from Germany and Switzerland showed the presence of 8 *Cryptosporidium* genotypes, with *C. parvum*, *C. hominis*, *C. muris*, and *C. andersoni* as the most prevalent species, and 4 samples having *C. baileyi* and 3 unidentified wildlife genotypes (Ward *et al.*, 2002). In a recent study conducted in the United Kingdom, all 14 finished water samples examined were positive for *C. hominis* by a new SSU rRNA-based PCR-RFLP tool (Nichols *et al.*, 2003). Results of these

recent studies support the conclusion that humans, farm animals, and wildlife all contribute to *Cryptosporidium* oocyst contamination in water.

Promising results in the genotyping of *Cryptosporidium* spp. in water samples have also been generated in recent studies using other techniques. HSP70 sequence analysis of cell culture-PCR amplified products revealed the presence of six sequence types of *C. parvum* in raw surface water samples and filter backwash water samples, all of which were from *C. parvum*, *C. hominis*, and *Cryptosporidium* mouse genotypes (Di Giovanni *et al.*, 1999), suggesting that farm animals, rodents, and humans were responsible for *Cryptosporidium* oocyst contamination in these waters. This was confirmed more recently in a more extensive study, in which infectious *C. parvum* and *C. hominis* oocysts were detected in 22 of 560 surface water samples, with *C. parvum* found in more than 90% of the positive samples (LeChevallier *et al.*, 2003). Analysis of six river water samples by a HSP70-based RT-PCR technique also showed the presence of *C. parvum* and *C. meleagridis* in two samples (Karasudani *et al.*, 2001). Using sequencing analysis of TRAP-C2, *C. parvum* was found in 11 of 214 surface and finished water samples in Northern Ireland in one study and in 2 of 10 river water and sewage effluent samples in another study (Lowery *et al.*, 2001a, 2001b). However, HSP70 and TRAP-C2-based primers are unlikely to amplify DNA of species genetically distant from *C. parvum* (Jiang and Xiao, 2003), and the primers used in the study by Karasudani *et al.* (2001) were previously shown to have poor specificity (Kaucner and Stinear, 1998).

4.5.3.2 Detection of *Cryptosporidium* Oocysts in Food Samples

The detection of parasites in food matrices has been a major challenge to parasitologists and food safety professionals for many years. First, there is a wide range of sample matrices. Second, the volume of materials needing to be analyzed is often huge when compared to the technical abilities of most traditional methods. Third, the load of parasites likely to be present is usually low. As a result, the recovery rate of detection methods for parasites in foodstuff can be very low (Bier, 1991).

The first step in the detection of *Cryptosporidium* oocysts in food after sampling is the elution of parasites from different food matrices. In the case of fruits, leafy greens or fresh produce, parasites can be recovered by washing the produce samples in 0.025M phosphate buffered saline, pH 7.25 (Ortega *et al.*, 1997). Sometimes, detergents (1% sodium dodecyl sulfate and 0.1% Tween 80, or the membrane filter elution buffer from EPA method 1623) and sonication (3–10 minutes) are also used to facilitate the elution of parasites from the food matrices (Bier, 1991; Robertson and Gjerde, 2000). The parasites are then concentrated by centrifugation and examined directly or after immunofluorescence staining (Bier, 1991; Ortega *et al.*, 1997). Sometimes, a sucrose floatation step is included to further purify parasites (Bier, 1991). Initially, this procedure was reported to have a low recovery rate of 1% for *Cryptosporidium* oocysts in cabbage and lettuce, two relatively simple food matrices (Bier, 1991). However, moderate recovery rates of 18.2–25.2% were subsequently reported for a variety of fresh produces (Ortega *et al.*, 1997). More recently, IMS has been used in the recovery of *Cryptosporidium* oocysts and *Giardia* cysts from fruits and vegetables, which has resulted in an improvement in recovery of parasites from lettuce, Chinese leaves, and strawberries to 42% for *Cryptosporidium* and 67% for

Giardia (Robertson and Gjerde, 2000). This new method includes washing procedures, sonication, IMS, immunofluorescence staining, and microscopy (Robertson and Gjerde, 2000, 2001a).

The detection of *Cryptosporidium* oocysts in shellfish is relatively easy compared to detection in vegetables, largely because the amount of materials for analysis is smaller and the number of oocysts potentially present is generally higher. In large molluscs such as oysters, mussels, and large clams, gills are usually removed with scissors, and washed by vortexing and centrifugation. *Cryptosporidium* oocysts present are examined and quantitated by microscopy after immunofluorescence staining. Sometimes, hemolymph is also harvested and *Cryptosporidium* oocysts in hemocytes are examined by immunofluorescence (Fayer *et al.*, 1998). With smaller molluscs such as small mussels and clams, the hemolymph or homogenized whole shellfish or gastrointestinal tract is generally examined individually or in pools (Graczyk *et al.*, 1999, 2001; Tamburrini and Pozio, 1999).

PCR has not been used in the analysis of fresh produce, but in theory, IMS-purified oocysts from fresh produces can be genotyped by molecular techniques using the same procedures developed for the analysis of water samples. Many studies have used PCR to genotype *Cryptosporidium* oocysts found in shellfish (Fayer *et al.*, 1999, 2002, 2003; Gomez-Bautista *et al.*, 2000; Gomez-Couso *et al.*, 2004; Graczyk *et al.*, 2001), which is useful in tracking the sources of contamination.

4.6 TREATMENT

Numerous pharmaceutical compounds have been screened for anti-*Cryptosporidium* activities *in vitro* or in laboratory animals. Some of those showing promise have been used in the experimental treatment of cryptosporidiosis in humans, but few have been shown to be effective in controlled clinical trials (Hunter and Nichols, 2002). Oral or intravenous rehydration is used whenever severe diarrhea is associated with *Cryptosporidium* infection (Hoepelman, 1996). Nitazoxanide (NTZ) is the only FDA approved drug for the treatment of pediatric cryptosporidiosis. It has also been recently approved for the treatment of Giardiasis in adult patients. Clinical trials have demonstrated that NTZ can shorten clinical disease and reduce parasite load (Amadi *et al.*, 2002; Rossignol *et al.*, 2001). This drug, however, is not yet approved for the treatment of *Cryptosporidium* infections in immunodeficient people, even though it is likely to be partially effective (Doumbo *et al.*, 1997; Rossignol *et al.*, 1998). For this population, paramomycin and spiramycin have been used in the treatment of some patients, but their efficacy remains unproven (Hewitt *et al.*, 2000). Thus, rehydration is still the major supportive treatment in AIDS patients (Hoepelman, 1996).

In industrialized nations, the most effective treatment and prophylaxis for cryptosporidiosis in AIDS patients is the use of highly active antiretroviral therapy (HAART) (Carr *et al.*, 1998; Miao *et al.*, 2000). Nonetheless, it is believed that the eradication and prevention of the infection is directly related to the replenishment of CD4+ cells in treated persons, rather than antiparasitic activities of these drugs (Carr *et al.*, 1998), even though some of the protease inhibitors used in HAART, such

as indinavir, nelfinavir, and ritonavir, have been shown to have anti-cryptosporidial activities *in vitro* and in laboratory animals (Hommer *et al.*, 2003; Mele *et al.*, 2003). Relapse of cryptosporidiosis is common in AIDS patients who have stopped taking HAART (Carr *et al.*, 1998; Maggi *et al.*, 2000).

4.7 CONTROL OF *CRYPTOSPORIDIUM* CONTAMINATION IN WATER AND FOOD

Cryptosporidium oocysts are very environmentally robust, with the capability for long-term survival in a variety of natural environments and resistance to most disinfectants. Unlike the majority of bacteria and viruses, *Cryptosporidium* spp. have an environmentally resistant resting stage in the form of the oocyst as part of its complex life cycle. The wall of oocysts allows the organism to remain viable for a considerable period, resist various harsh environmental challenges, and await the opportunity to infect a new susceptible host. Table 4.4 summarizes the survival of *Cryptosporidium* oocysts in a variety of matrices under controlled conditions in selected studies. *Cryptosporidium* oocysts can survive for months in soil, fresh water, and seawater. Thus, natural contamination of the environment can accumulate over time and the contaminated environment may be a reservoir of viable oocysts for long periods of time. For example, Tamburrini and Pozio (1999) reported that oocysts remain infective in seawater for up to one year and can be filtered out by benthic mussels, which retain their infectivity.

A combination of filtration and disinfection is required for controlling *Cryptosporidium* oocysts in water, which also helps to reduce the contamination in foods and beverages. Physical removal of oocysts from drinking water through coagulation, sedimentation, and filtration is the primary defense against waterborne cryptosporidiosis (Rose, 1997). Deficiencies in any one of these processes have been shown to directly affect the efficiency of overall oocyst removal (Medema *et al.*, 2003). Properly operated conventional treatment (coagulation/floccation, sedimentation, filtration, and disinfection) can remove 99% or more of oocysts (Hashimoto *et al.*, 2001; Hijnen *et al.*, 2004; Hsu and Yeh, 2003). One of the critical times when oocysts can breach the filtration barrier is following backwash (Karanis *et al.*, 1996). For this reason, optimization of the backwash procedure, including the addition of coagulants, or filtering of waste can minimize the passage of oocysts.

Chlorination alone has not been successful in eliminating waterborne *Cryptosporidium* oocysts. As much as 80mg/l of free chlorine or monochloramine requires 90 minutes to produce 90% oocyst inactivation (Korich *et al.*, 1990). Chlorine dioxide, on the other hand, seems to be more effective than free chlorine. Peeters *et al.* (1989) reported that 0.43mg/l of chlorine dioxide (ClO_2) reduced infectivity within 15 min, although some oocysts remained viable. Korich *et al.* (1990) reported approximately 90% inactivation of oocysts exposed to 1.3mg/l of chlorine dioxide for 60 min. In contrast, ozone and ultra violet (UV) radiation have shown the most promise as effective inactivation practices. An initial concentration of 1.11mg/l ozone for 6 min was shown to inactivate viable oocysts at a concentration of 10^4 oocysts/ml (Peeters *et al.*, 1989). Korich *et al.* (1990) reported that exposure

Table 4.4. Percentage reduction in *Cryptosporidium* oocyst viability with different treatments.

	Treatment	*%Reduction**	*Reference*
Water	60 days at natural condition (*C. parvum*)	54	(Kato *et al.*, 2001)
	120 days at natural condition (*C. parvum*)	89	(Kato *et al.*, 2001)
Soil	60 days at natural condition (*C. parvum*)	61	(Kato *et al.*, 2001)
	120 days at natural condition (*C. parvum*)	90	(Kato *et al.*, 2001)
Silage	106 days (*C. parvum*)	46–62	(Merry *et al.*, 1997)
Mineral water	4°C for 12 weeks (*C. parvum*)	1–11	(Nichols *et al.*, 2004)
	20°C for 12 weeks (*C. parvum*)	22–59	(Nichols *et al.*, 2004)
	4·5% NaCl at 22°C for 8 days (*C. hominis*)	77	(Dawson *et al.*, 2004)
	4·5% NaCl 9 days at 22°C (*C. parvum*)	57	(Dawson *et al.*, 2004)
	9% Ethanol 7 days at 22°C (*C. hominis*)	77	(Dawson *et al.*, 2004)
	9% Ethanol 8 days at 22°C (*C. hominis*)	66	(Dawson *et al.*, 2004)
	40% Ethanol 8 days at 22°C (*C. hominis*)	72	(Dawson *et al.*, 2004)
Food and beverage treatment	20% Glycerol 7 days at 4°C (*C. hominis*)	57	(Dawson *et al.*, 2004)
	20% Glycerol 13 days at 4°C (*C. parvum*)	85	(Dawson *et al.*, 2004)
	20% Glycerol 13 days at 22°C (*C. parvum*)	87	(Dawson *et al.*, 2004)
	20% Glycerol 14 days at 4°C (*C. parvum*)	53	(Dawson *et al.*, 2004)
	50% Sucrose 7 days at 22°C (*C. hominis*)	100	(Dawson *et al.*, 2004)
	50% Sucrose 8 days at 22°C (*C. hominis*)	86	(Dawson *et al.*, 2004)
	50% Sucrose 9 days at 22°C (*C. parvum*)	90	(Dawson *et al.*, 2004)

(*continued*)

Table 4.4. (*continued*)

	Treatment	*%Reduction**	*Reference*
Water and water treatment	Frozen at −22°C for 297 h (*C. parvum*)	86	(Robertson *et al.*, 1992)
	4°C for 176 days (*C. parvum*)	57–66	(Robertson *et al.*, 1992)
	In seawater at 4°C for 35 days (*C. parvum*)	22–31	(Robertson *et al.*, 1992)
	1.5 ppm aluminum at room temperature for 7 mins (*C. parvum*)	3–4	(Robertson *et al.*, 1992)
	16 ppm ferric sulfate at room temperature for 1 h (*C. parvum*)	37	(Robertson *et al.*, 1992)
	0.2% calcium hydroxide (lime) at room temperature for 1 h (*C. parvum*)	30	(Robertson *et al.*, 1992)
	250–270 nm UV radiation at 2 mJ/cm^2 (*C. parvum*)	1.8–2.3 log	(Linden *et al.*, 2001)

to 1mg/l ozone inactivated between 90 and 99% of oocysts (2.8×10^5/ml) in water at 25°C. Ninety-nine to 99.9% inactivation was achieved when the exposure time was increased to 10 mins. In addition to ozone treatment, UV radiation has now been rapidly adopted by the water industry for inactivation of *Cryptosporidium* oocysts in water (Bukhari *et al.*, 2004; Lorenzo-Lorenzo *et al.*, 1993). UV light between 250 and 270 nm in wavelengths have been shown to reduce *C. parvum* oocyst infectivity at 2 mJ/cm^2 (Linden *et al.*, 2001). Higher doses can lead to higher inactivation rates (Craik *et al.*, 2001). Most chemicals used in floccation during the first step of water treatment have only a limited effect on the viability of *Cryptosporidium* oocysts at the practical concentrations (Robertson *et al.*, 1992).

Cryptosporidium oocysts can contaminate food through many pathways. These include (i) introduction to the foodstuff through contaminated raw ingredients, e.g., unwashed lettuce destined for ready-to-eat salads; (ii) introduction during food processing due to addition of contaminated water, as an important ingredient of the foodstuff, e.g., in soft drinks production; (iii) introduction during food processing as a contaminant of equipment cleaning with non-potable water; (iv) introduction of the parasite through pest infestations, e.g., cockroaches, house flies, mice, and rats; and (v) introduction of the parasite to processed foodstuffs from positive food handlers. The associated risk from each of these potential routes of entry of oocysts into the foodstuff should be controlled through an integrated HACCP (hazard analysis and critical control point) management.

The effect of food processing and storage practices on the viability of potentially contaminated *Cryptosporidium* oocysts in food and beverage depends on the nature of the treatment. Snap freezing is detrimental to the survival of *Cryptosporidium*

oocysts (Fayer and Nerad, 1996; Robertson *et al.*, 1992), but if suspended in water, stored at −20° C for 24 h, and then transferred to −70°C, *C. muris* oocysts can survive for at least 15 months (Rhee and Park, 1996). Some *C. parvum* oocysts can survive freezing in water at higher temperatures (−20–22°C) for 1–32 days (Deng and Cliver, 1999a; Fayer and Nerad, 1996; Robertson *et al.*, 1992). As expected, air drying for 4 h kills almost all *C. parvum* oocysts (Deng and Cliver, 1999a; Robertson *et al.*, 1992). *Cryptosporidium* oocysts can survive high temperatures for only short durations. Oocysts of *C. parvum* lose infectivity at 72.4°C or higher within 1 min, or when the temperature is held at 64.2°C or higher for 2 min (Fayer, 1994). The high-temperature-short-time conditions (71.7°C for 15 s) used in commercial pasteurization are sufficient to destroy infectivity of *C. parvum* oocysts in milk and apple cider (Harp *et al.*, 1996; Deng and Cliver, 2001). Most oocysts of *C. parvum* can survive for at least 10 days during the process of yogurt making and storage, but cannot survive the ice cream making process (Deng and Cliver, 1999a).

Not much is known on the survival of *Cryptosporidium* oocysts in beverage. Although high salinities reduce the survival of *C. parvum* in water (Fayer *et al.*, 1998), oocysts can maintain viability for months in natural mineral water, especially at low temperatures (Nichols *et al.*, 2004). There is some reduction in oocyst viability in acidified and carbonated beverages (Friedman *et al.*, 1997). Oocysts of *C. hominis* stored in 9 or 40% ethanol for 7 or 8 days at 22°C suffer 66–77% reductions in viability (Dawson *et al.*, 2004). However, their ability for long-term survival in ethanol is not clear.

Not all food preservatives have detrimental effects on the viability of *Cryptosporidium* oocysts. Considerable viability is maintained when *C. parvum* oocysts are stored at 4°C or 22°C in media containing citric, acetic, or lactic acid (Dawson *et al.*, 2004). Oocysts of *C. parvum* and *C. hominis* kept in 4.5% sodium chloride at 22°C for 8 or 9 days have 57–77% reduction in viability. Similar losses in viability also occur in oocysts stored in 20% glycerol at 4°C or 22°C for 13 or 14 days. Storage in 50% sucrose at 22°C, however, is detrimental to most *C. parvum* and *C. hominis* oocysts (Dawson *et al.*, 2004).

Presently, there is no solid recommendation regarding the management of *Cryptosporidium*-positive food handlers within the food-processing sector. The mean duration of the illness has previously been reported as 12.2 days; however, the range in duration is 2 to 26 days (Jokipii and Jokipii, 1986). Oocyst excretion times have varied widely from 6.9 days (range 1–15 days) after the cessation of symptoms, to 2 months and greater in a small proportion of patients. Thus, it is impossible to predict the carrier status of persons based on cessation of symptoms. In addition, microbiological screening for carrier status in infected persons is problematic as symptomatic patients may have intermittently negative stool specimens (Jokipii and Jokipii, 1986). Other studies have shown that asymptomatic carriers are found in 0.4% of the general population in Australia (Hellard *et al.*, 2000) and 6.4% of immunocompetent children in the United States (Pettoello-Mantovani *et al.*, 1995). Thus, consumers of ready-to-eat foodstuffs are vulnerable to potential contamination of products by food-handlers with both symptomatic and asympotomatic cryptosporidiosis (Quiroz *et al.*, 2000). Therefore, it is important that other general hygienic practices such as hand washing and glove wearing are also implemented as

part of the HACCP management to minimize food poisoning due to cryptosporidiosis and other pathogens.

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