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The Epidemiology of *Cryptosporidium* Species in a Nigerian Paediatric Population

Síle F. Molloy

A thesis submitted in the fulfilment for the Degree of Doctor in Philosophy to
Trinity College, University of Dublin

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Summary

Cryptosporidium is an Apicomplexan, protozoan parasite, infecting the gastroepithelium of many vertebrate hosts, including man, and is the causative agent of the diarrhoeal disease cryptosporidiosis. Although infection may be asymptomatic or mild and self-limiting in immunocompetent patients, in the immunocompromised (especially the malnourished and those with HIV/AIDS), the disease can be severe, leading to chronic dehydration and even death (Dillingham *et al.*, 2002).

The prevalence of infection varies both geographically, between the developed and the developing world, and possibly also temporally, between the rainy and dry seasons. Recently, attention has focused on childhood disease, as studies have indicated that children are at high risk for infection (particularly those under the age of 5 years) (Nimri and Hijazi, 1994; Bhattacharya *et al.*, 1997; Chacin-Bonilla *et al.*, 1997; Iqbal *et al.*, 1999; Newman *et al.*, 1999; Abdel-Messih *et al.*, 2005). It has been suggested that cryptosporidiosis may have long-term negative effects on children's growth and cognitive development (Guerrant *et al.*, 1999; Tumwine *et al.*, 2003). Several species, genotypes and subtypes of *Cryptosporidium* that infect humans have been identified, with variations for each regarding source (host range), transmission routes, pathogenicity and risk factors for infection (Hunter *et al.*, 2004; Cama *et al.*, 2007; Lake *et al.*, 2007; Cama *et al.*, 2008).

Population-based epidemiological data on high-risk paediatric groups in developing countries is lacking, especially in relation to the species, genotypes and subtypes of *Cryptosporidium* present. Therefore, this thesis represents an investigation of the prevalence, temporal variability and molecular epidemiology of *Cryptosporidium* in Nigerian children.

The study was conducted in 4 semi-urban villages, within 10km of Ile-Ife, Osun State, Nigeria. Stool samples were collected from a total of 1636 children at 4 time points over a 1 year period. Stool samples were tested for the presence of *Cryptosporidium* oocysts using fluorescent microscopy (formal-ether concentration followed by auramine-phenol staining). Prevalence of infection ranged from 15.6% in September 2006 to 19.6% in May 2007 and the vast majority of positive samples were of low intensity. A total of 349 children provided samples on all 4 occasions and using gllamm analysis (Rabe-Hesketh *et al.*, 2002), month was statistically significantly associated with infection, with increased risk in both May

(OR 1.11, 95% CI: 1.049-1.178, P<0.0001) and February (OR 1.06, 95% CI: 1.003-1.127, P=0.034) in a model adjusted for age, gender and village of residence. There was no statistically significant association between infection status and age, gender or village of residence of the children.

Genotyping data for 77 positive samples was obtained using nested polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) of 2 loci at the small subunit (SSU) rRNA gene fragment. The following species were identified: *Cryptosporidium hominis* (34), *Cryptosporidium parvum* (25), *C. parvum / C. hominis* (4), *Cryptosporidium meleagridis* (5), *Cryptosporidium* rabbit genotype (5), *Cryptosporidium* cervine genotype (3) and *Cryptosporidium canis* (1). Glycoprotein 60 (GP60) subtyping revealed that 28 amplifiable *C. hominis* isolates consisted of 5 subtypes (Ia, Ib, Id, Ie and 1 novel subtype (Ih), belonging to 12 subtype allele families) and 23 amplifiable *C. parvum* isolates consisted of 4 subtypes (IIa, IIc, IIi and 1 unnamed subtype, belonging to 6 subtype allele families). Three of 5 isolates of *C. meleagridis*, subtyped by sequence analysis of the SSU rRNA gene fragment were *C. meleagridis* Type 1.

Risk factors for sporadic *Cryptosporidium* infection were investigated by means of a cross-sectional study. A binomial generalised linear model identified a number of risk factors for infection. For all *Cryptosporidium* infections, a positive association was identified between infection with *Cryptosporidium* and malaria infection, while a negative association was identified between *Cryptosporidium* infection and *Ascaris* infection. In addition, children that had been breast-fed exclusively had a decreased risk of infection. An interaction between crowding and the presence of a member in the household with diarrhoea was also statistically significant. Furthermore, risk factors varied by species with younger age, decreasing levels of maternal education and increased severity of stunting associated with risk for *C. hominis* infections, while for *C. parvum* infections, risk was associated with increased severity of stunting and malaria infection.

To our knowledge, this is the first study conducted in Nigeria to determine the species, genotypes and subtypes present in a paediatric population and results highlight a high diversity of species present in the population. Species data from this high-risk paediatric group coupled with risk factors for infection will greatly add to our knowledge of the epidemiology of *Cryptosporidium* in developing countries.

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Table of Contents

1 Introduction to *Cryptosporidium* species

Section **Page**

1.1 Introduction

Cryptosporidium was first described in detail by Tyzzer (1907) when he extracted the parasite from the gastric epithelium of mice. The first species was named *Cryptosporidium muris* and in 1912 a second species, *Cryptosporidium parvum*, was also identified in mice (Tyzzer, 1912). In 1955 a third species, *Cryptosporidium meleagridis*, was reported in turkeys (Slavin, 1955). However, the parasite was not detected in humans until 1976 when it was reported in a 3-year-old immunocompetent child (Nime *et al.*, 1976) and later that year in a 39-year-old immunosuppressed man with diarrhoea (Meisel *et al.*, 1976). According to Xiao and Fayer (2008), prior to the 1980s less than 100 reports on cryptosporidiosis had been published. However, by the end of the 1980s, there were over 1200 reports. The increase in interest in the disease coincided with the AIDS epidemic when it was discovered that cryptosporidiosis was a life-threatening disease in AIDS patients (Anonymous, 1982; Ma and Soave, 1983).

Public awareness of the disease increased in 1993 when a large-scale waterborne outbreak of cryptosporidiosis occurred in Milwaukee, USA, infecting over 400,000 people (Mackenzie *et al.*, 1994). This incident highlighted *Cryptosporidium* as an important human enteric pathogen. Owing to its environmental robustness and the current lack of effective treatment, cryptosporidiosis has become one of the most important causes of diarrhoea worldwide. In addition, asymptomatic infections have been identified in an increasing number of healthy individuals (Esteban *et al.*, 1998; O'Donoghue, 1995).

1.2 Taxonomy

Cryptosporidium is a minute Apicomplexan, protozoan parasite, belonging to the class Sporozoasida (Tzipori and Ward, 2002). All species are obligate parasites infecting the micro-villus border of the gastrointestinal epithelium (Fayer *et al.*, 2000; Bush *et al.*, 2001; Fayer, 2004) of a wide range of vertebrate hosts, including humans (Xiao *et al.*, 2004). Over 150 mammalian hosts as well as birds, reptiles, amphibians and fish have been found to be parasitised by the genus *Cryptosporidium* (Fayer, 2008). This parasite was once thought to be related to

the Coccidia (intracellular parasites of the gut and other organs of vertebrates) but it is now widely believed that *Cryptosporidium* should be placed in a taxonomic grouping separate from the Coccidia and closer to the Gregarines (extra and/or intra-cellular protozoan parasites) (Barta and Thompson, 2006). *Cryptosporidium* species are morphologically similar to the Coccidia throughout their life cycle, however, according to Barta and Thompson (2006), this species has always been considered an atypical coccidian due to the autoinfective oocysts (ability to establish a life cycle within the host), their lack of possession of a mitochondrion-like organelle [despite the presence of mitochondrion-specific genes (Riordan *et al.*, 1999)] and their insensitivity to anticoccidial drugs. Previously, it was thought that *Cryptosporidium* was largely an intracellular parasite, however the occurrence of predominantly extracellular stages in the life cycle which can be completed in cell-free culture has recently been demonstrated (Barta and Thompson, 2006). This lack of clarity in relation to the taxonomy of *Cryptosporidium* has presented challenges for researchers. Without a defined taxonomic grouping, understanding the transmission dynamics and therefore the public health significance of the parasite is limited.

According to Fayer (2004), it was once believed that *C. parvum*, a single species, infected 155 mammalian hosts including humans, however, several species have now been identified which are associated with different hosts. *Cryptosporidium* species were initially distinguished by their oocyst size, host specificity and site of infection within the animal (with species often being named after the host in which they were discovered e.g. *Cryptosporidium canis* was first discovered in the dog). Presently, molecular techniques have been used to establish a genetic basis for these distinctions (Ong *et al.*, 1999; Xiao *et al.*, 1999b; Xiao *et al.*, 1999a; Guyot *et al.*, 2001).

The development of molecular techniques was required for a number of reasons. Firstly, *Cryptosporidium* appear to have some host specificity but are not strictly host specific e.g. *C. parvum* has been identified in mice, cattle, humans, horses and other mammalian hosts (Fayer, 2004) and *Cryptosporidium baileyi*, *C. canis*, *Cryptosporidium felis*, *C. meleagridis* and *C. muris*, once all thought to be host specific (*C. baileyi* in chickens, *C. canis* in dogs, *C. felis* in cats, *C. meleagridis* in turkeys and *C. muris* in mice) have all been found to infect humans (Ditrich *et al.*, 1991; Guyot *et al.*, 2001; Xiao *et al.*, 2001a; Caccio *et al.*, 2005). In addition, *Cryptosporidium hominis*, once thought to exclusively infect humans, has been subsequently found in other vertebrate hosts (Morgan *et al.*, 2002; Park *et al.*, 2006). Secondly, morphology of oocysts alone cannot be used to identify most of

the species, as oocysts are often almost identical in size and similar in structure. Genetic characterisation of *Cryptosporidium* species has allowed researchers to define relationships among parasite species, potential hosts and pathways of transmission (Xiao *et al.*, 2004). It has been suggested that 4 basic requirements be fulfilled when naming new *Cryptosporidium* species: (1) morphology of oocysts, (2) genetic characterisation, (3) demonstration of natural, and where possible, some experimental host specificity; and (4) compliance with the ICZN (International Code of Zoological Nomenclature) (Xiao *et al.*, 2004).

As discussed by Xiao and Fayer (2008), some isolates which were thought to have been *C. parvum* have since been elevated to species level (*C. hominis*, *C. bovis* and *C. suis*), while other isolates have been differentiated at the molecular level (*C. parvum*, *C. hominis*, *C. canis*, *C. galli* and *C. muris*) and are now genotypes or sub-genotypes (subtypes). Currently there are 21 recognised species of *Cryptosporidium* (Table 1.1) and over 40 genotypes (Table 1.2). Of these, at least 8 species and 7 genotypes have been found to infect humans (Table 1.3). Although many studies have shown that it is predominantly *C. hominis* and *C. parvum* which infect humans, Guyot *et al.* (2001) indicated that immunocompromised individuals are susceptible to a wide range of *Cryptosporidium* species and genotypes including *C. meleagridis*, *C. felis*, *C. canis* and *C. muris*. However, there is increasing evidence that both immunocompetent and immunosuppressed individuals can be infected with this wide range of species (Xiao *et al.*, 2001a; Gatei *et al.*, 2006a).

Table 1.1 Valid named species of *Cryptosporidium* [modified from Fayer, (2008) and Xiao and Fayer (2008)]

Species	Oocyst dimensions (µm)	Site of Infection	Major hosts	Minor hosts	Author
C. andersoni	6.0-8.1 x 5.0-6.5	Abomasum	Cattle, Bactrian camel	Sheep, humans	Lindsay *et al.* (2000)
C. baileyi	5.6-6.3 x 4.5-4.8	Bursa	Chickens	Quail, ostriches, ducks	Current *et al.* (1986)
C. bovis	4.8-4.5 x 4.2-4.8	Small intestine	Cattle	Sheep	Fayer *et al.* (2005)
C. canis	4.9 x 4.7	Small intestine	Dogs	Human	Fayer *et al.* (2001)
C. fayeri	4.5-5.1 x 3.8-5.0	Small intestine	Red kangaroo	Unknown	Ryan *et al.* (2008)
C. felis	5.0 x 4.5	Small intestine	Cats	Humans, cattle	Iseki (1979)
C. fragile	5.5-7.0 x 5.0-6.5	Stomach	Toads	Unknown	Jirku *et al.* (2008)
C. galli	8.0-8.5 x 6.2-6.4	Proventriculus	Chickens, finches	Unknown	Pavlasek (1999)
C. hominis	4.4-5.9 x 4.4-5.4	Small intestine	Humans	Dugong, sheep, cattle	Morgan *et al.* (2002)
C. macropodum	4.5-6.0 x 5.0-6.0	Unknown	Grey kangaroo	Unknown	Power and Ryan (2008)
C. meleagridis	4.5-6 x 4.2-5.3	Small intestine	Turkeys, humans	Parrots	Slavin (1955)

Table 1.1 Contd

Species	Oocyst dimensions (µm)	Site of Infection	Major hosts	Minor hosts	Author
C. molnari	3.2-5.5 x 3.0-5.0	Stomach and intestine	Fish (Sea bream, Sea bass)	Unknown	Alvarez-Pellitero and Sitja-Bobadilla (2002)
C. muris	7.5-9.8 x 5.5-7.0	Stomach	Rodents	Humans	Tyzzer (1907)
C. parvum	4.5-5.4 x 4.2-5.0	Small intestine	Cattle, humans	Deer, mice, pigs	Tyzzer (1912)
C. ryanae (previously *Cryptosporidium* deer-like genotype)	2.9–4.4 x 2.9–3.6	Not known	Cattle	Not known	Fayer *et al.* (2008)
C. scophthalmi	3.7-5.0 x 3.0-4.7	Intestine and stomach	Fish (Turbot)	Unknown	Alvarez-Pellitero *et al.* (2004)
C. serpentis	5.5-6.6 x 4.8-5.6	Stomach	Snakes, lizards	Unknown	Brownstein *et al.* (1977)
C. suis	4.4-4.9 x 4.0-4.3	Small and large intestine	Pigs	Humans	Ryan *et al.* (2004)
C. varanii	4.8-5.1 x 4.4-4.8	Small and large intestine	Lizards	Snakes	Pavlasek *et al.* (1995)
C. wrairi	4.8-5.6 x 4.0-5.0	Small intestine	Guinea pigs	Unknown	Vetterling *et al.* (1971)
C. xiaoi	2.9–4.4 x 2.9–4.4	Unknown	Sheep	Yak, goat	Fayer and Santin (2009)

Table 1.2 *Cryptosporidium* genotypes [modified from Fayer (2008)]

***Cryptosporidium* genotypes**		
Bear	Horse	Pig II
C. canis Coyote; Dog; Fox	Lizard	Rabbit
Caribou	*C. muris* Japanese field mouse	Raccoon
Cervine	Marsupial I and II	Seal 1 and 2
Deer	Mongoose	Sheep novel genotype
Deer mice	Monkey	Skunk
Duck	Mouse	Snake
Ferret	Muskrat I and II	Squirrel
Fox and Fox II	Opossum I and II	Tortoise
C. galli finch	Ostrich	Woodcock
Goose I and Goose II	Ovine	

Table 1.3 *Cryptosporidium* spp. and genotypes that infect humans and other hosts [modified from Xiao and Fayer (2008)]

Species	**Hosts**
C. andersoni	Cattle, sheep, Bactrian camel, gerbil, multimammate mouse, wood partridge
C. baileyi	Chicken, duck, Bobwhite quail (not identified conclusively in humans)
C. canis	Dog, fox, coyote
C. felis	Cat, cattle
C. hominis	Primates, cattle, sheep, pig, dugong
C. meleagridis	Turkey, chicken, Bobwhite quail, dog, deer, mouse
C. muris	Mouse, hamster, squirrel, Siberian chipmunk, Wood mouse, Bank vole, Rock hyrax, Bactrian camel, Mountain goat, cat, coyote, Ringed seal, bilby, Cynomolgus monkey, Tawny frogmouth
C. parvum	Calf, lamb, horse, alpaca, dog, mouse, raccoon dog, Eastern grey squirrel
C. suis	Pig, cattle
Cervine genotype	Cattle, sheep, ibex, Eastern grey squirrel, chipmunk, beaver, red squirrel, woodchuck, deer mouse, raccoon, deer, mouflon sheep, blesbok, nyala, lemur
Chipmunk genotype I	Chipmunk, Eastern grey squirrel, deer, mouse
Horse genotype	Horse
Monkey genotype	Monkey
Pig genotype II	Pig
Skunk genotype	Skunk, raccoon, Eastern grey squirrel, opossum, River otter
Rabbit genotype	Rabbit

1.3 Biology

1.3.1 Life cycle

Cryptosporidium oocysts represent both the infective and the diagnostic stage of the life cycle and are released in the host faeces. They are transferred to another host via routes such as contaminated drinking water/food or direct contact with infected faeces.

Each oocyst contains 4 naked sporozoites. Following ingestion these sporozoites are released within the host in response to body temperature, acid trypsin and bile salts (Smith *et al.*, 2005a). The sporozoites penetrate the mid gut epithelial cells and differentiate into trophozoites. All life cycle stages occur in the epithelial cells within parasitophorous vacuoles situated in the brush border between the plasma membrane and the cytoplasm or in the lumen (Smith and Grimason, 2005). The trophozoites undergo nuclear proliferation to form Type I meronts. This Type I meront undergoes 3 nuclear divisions releasing 6 to 8 merozoites which leave the meront to form either a Type I meront or a Type II meront. A Type II meront will undergo 2 nuclear divisions and release 4 merozoites which go on to form the micro- and macro- gametes. The microgamete fuses with the macrogamete to form a zygote (the oocyst). Oocysts sporulate *in-situ*. Approximately 20% of oocysts are thin-walled and involved in autoinfection, while the remaining 80% pass from the body in the faeces. The latter are acid-fast, thick-walled oocysts and, once released into the environment, go on to infect a new host. Sporozoites, produced from thin-walled oocysts, can excyst while still within the gut. Therefore, *Cryptosporidium* appears to have 2 autoinfective cycles: the first by continuous recycling of Type I meronts and the second through sporozoites excysting from thin-walled oocysts (Figure 1.1).

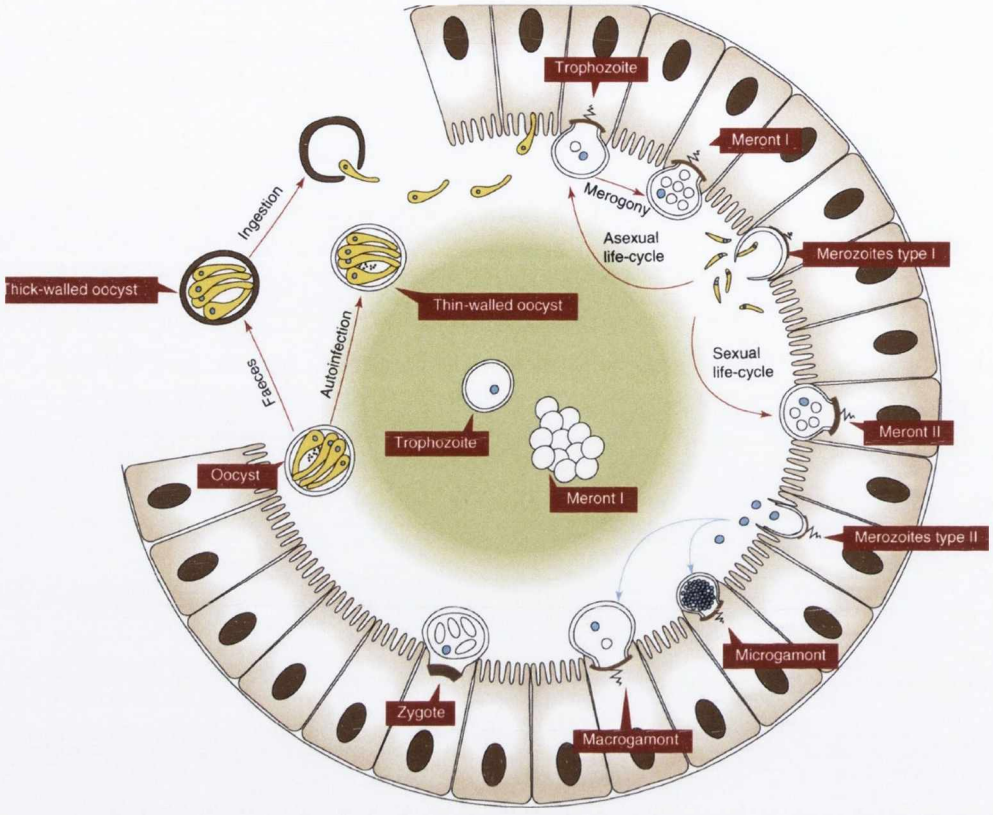

Figure 1.1 Life cycle of *Cryptosporidium* sp.
Adapted from Borowski *et al.* (2008)

1.3.2 Oocyst morphology

The oocyst is the infective stage of the life cycle and due to its hardy outer wall, is environmentally robust. The size of the oocyst is species dependent and ranges from 3.2 x 3.0 microns (*C. molnari*) to 8.5 x 6.4 microns (*C. galli*) (Table 1.1). The *C. parvum* oocyst consists of a trilaminar wall, with an average thickness of ~49 nm (Harris and Petry, 1999). The outer layer is made up of a glycoprotein and averages 10 nm in thickness. The middle layer is a 2.5 nm thick glycolipid / lipoprotein and is thought to be responsible for the acid-fast staining property of the wall (Harris and Petry, 1999). The third layer, also thought to be a glycoprotein, provides rigidity and elasticity and is composed of an inner (11.6nm) and outer (25.8nm) layer (Harris and Petry, 1999). Beneath this trilayered wall is a membrane-enclosed residual body and 4 naked sporozoites (Smith *et al.*, 2005a). During excystation, the oocyst wall retracts (in response to body temperature, acid trypsin and bile salts (Smith *et al.*, 2005a)) and the sporozoites are released.

The oocyst is important for the dispersal, survival and infectivity of the parasite and the lack of an effective agent for control contributes to its pathogenic potential and public health significance.

1.3.3 Environmental robustness

Owing to the hardy outer wall, *Cryptosporidium* oocysts are extremely robust and can remain viable and infective in the environment for long periods of time. The effect of various types of stress such as temperature, salinity, desiccation and pH on oocyst viability have been investigated and the ability of the oocysts to withstand these pressures may have major implications for the survival and transmission of the parasite.

1.3.3.1 Temperature

At very low and very high temperatures, oocysts will lose infectivity. Snap freezing and freezing at -70°C immediately kills the oocyst (Fayer and Nerad, 1996; Robertson *et al.*, 2002). According to Fayer and Nerad (1996), oocysts frozen at -20°C for 1, 3 and 5h were infective in mice but oocysts stored at -20°C for 24h and 168h were not infective. This indicates that a combination of both temperature, and time spent at a particular temperature, play an important role in decreasing infectivity. Oocysts stored at -15°C for 168h showed no infectivity but those kept at -15°C for 8h and 24h, -10°C for 8h, 24h and 168h and 5°C for 168h were all infective in mice. These findings are in contrast to those of Robertson *et al.* (1992) who showed that after 21h at -22°C, 67% of oocysts were killed and 79% killed after 152h. It was also found that after 750h at -22°C a small proportion of oocysts were still viable.

Fayer *et al.* (1998a) demonstrated that oocysts held at 10°C were infectious for up to 1 week of storage and those at -5°C were infectious for up to 2 months. In addition, the effect of repeated freeze-thaw at -10°C on oocysts in soil with 3-78% water did not significantly differ from those held at -10°C under static conditions (Kato *et al.*, 2002) indicating that repeated freeze-thawing in the environment will not affect viability of oocysts unless the temperature is low enough. Although results vary slightly among studies, the general trend implies that freezing below -20°C significantly reduces the viability of oocysts and the longer they are frozen the lower the risk of infectivity.

In relation to oocysts held at elevated temperatures, Fayer *et al.* (1998a) found that oocysts stored in deionised water at 0, 5, 10, 15 and 20°C were infectious after 6 months with oocysts held at 20°C infecting 1 of 10 mice after 3 months. If held at 25 and 30°C, oocysts infected 10% of mice (n=10) for up to 3 months and at 35°C, light infection was observed in 20% of mice after 1 week. Fayer (1994) indicated that oocysts were non-infectious after 1 minute at 72.4°C and for 2 minutes at 64.2°C. Jenkins *et al.* (1999) suggests that the increase in death rate at higher temperatures may be owing the destruction of hydrocarbons in the oocyst wall with melting points of 18-60°C. This would allow sporozoites to be exposed to environmental pressures thereby killing the parasite. Generally it appears that at temperatures below -20°C and above 20°C, survival times for oocysts will be significantly reduced. Although data regarding oocyst survival in tropical countries is lacking, it is likely that oocysts will survive in such environments and so risk of infection in these areas may be high.

1.3.3.2 Salinity

Viability of oocysts in sea water may be important for parasite transmission. If oocysts have the ability to withstand saline conditions then disposal of human waste into the sea and harvesting of food from the same source could present an opportunity for parasite transmission. Fayer *et al.* (1998b) found that oocysts in salinities of 10, 20 and 30ppt held at 10°C and 10ppt at 20°C were still infective after 3 months. In water at 20°C and salinities of 20ppt and 30ppt oocysts were infectious for 8 and 4 weeks, respectively. This study also found that oysters harboured infectious *C. parvum* oocysts and so these animals may serve as mechanical vectors for transmission of the parasite. Tamburrini and Pozio (1999) showed that oocysts remain viable and infective to mice after 12 months in seawater at 6-8°C. The survival of oocysts in saline conditions is consistent with other studies (Fayer *et al.*, 1998a; Freire-Santos *et al.*, 1999; Robertson *et al.*, 2002). However, the effect of salinity on oocysts viability seems to be less important than the effects of temperature and length of time spent under those temperatures. Consequently, the ability of oocysts to survive under saline conditions suggests that the risk of contracting cryptosporidiosis from sea water filter feeders cannot be overlooked.

1.3.3.3 Desiccation

Desiccation was shown to reduce the viability of oocysts. 70% were killed after 2 hours of air drying and 100% killed after 4 hours (Robertson *et al.*, 1992). When

oocysts were submerged in human stool samples, results for oocysts viability varied (Robertson *et al.*, 1992). Although only 3 human stool samples were tested in the study, the general trend indicated that after 48 days there was a decrease in viability of oocysts. The authors suggest that oocysts which come in contact with faeces develop an enhanced impermeability to small molecules which may increase robustness when exposed to environmental pressures.

1.3.3.4 pH

Both very low and very high pH levels will decrease the viability and infectivity of oocysts while neutral pH levels will have little effect. Robertson *et al.* (1992) subjected oocysts to high concentrations of lime for long periods which resulted in a significant increase in oocyst death. When pH was changed from 10.5 to 6, the viability was not altered. Likewise, high concentrations of ferric sulphate also significantly increased oocyst mortality and when pH was changed from 1.5 to 6 there was no impact on viability. Other studies provided evidence that pH may not significantly influence the viability of oocysts (Dawson *et al.*, 2004). Friedman *et al.* (1997) suggested that oocysts can survive in soils with low pH but survival decreased with time.

Although a range of factors affect the viability and infectivity of *Cryptosporidium* oocysts, it is generally extremes (not usually found under regular environmental conditions) in these factors that will render the oocysts inactive. Therefore, the hardy oocysts are extremely environmentally robust, leading to increased potential for transmission, thus, enabling the parasite to thrive. Understanding the limits of the oocysts' resistance helps researchers to implement programmes (e.g. in water treatment works) which limit the spread of the parasite.

1.4 Infection in humans

1.4.1 Host-parasite interaction

The mechanisms of host cell penetration by *Cryptosporidium* and subsequent infection is poorly understood. Infection occurs when oocysts are ingested and sporozoites are released which attach to and invade the intestinal epithelial cells

(Leav *et al.*, 2002b). The jejunum and the ileum are the most common sites of infection in humans, however in AIDS patients, the stomach, duodenum, colon and bilary tract may also be infected (Hunter and Nichols, 2002). Once attached to the epithelial wall of the gastrointestinal tract, the parasite is protected from the hostile environment of the gut and the host's immune system. A parasitophorous vacuole surrounds the parasite and is connected to the host cell by a parasitophorous vacuole membrane (Tzipori and Ward, 2002). Here, the parasite gains energy and nutrients from the host cell through a unique feeder organelle. This organelle directly separates the cell and parasite cytoplasm (Tzipori and Ward, 2002).

According to Thompson *et al.* (2005), apical complex proteins (Glycoprotein 900 and 400) facilitate the attachment of the sporozoite. This is accomplished through ligand-host receptor interactions which lead to invasion and formation of the parasitophorous vacuole on the epithelial surface (Thompson *et al.*, 2005). When the parasite enters the apical surface of the epithelium cells it rests on a bed of host actin filaments and actin-binding proteins. Actin-polymerising factors are then involved in the reorganisation of the host cytoskeleton (Sibley, 2004). According to Sibley (2004), *Cryptosporidium* exhibits actin dependent 'gliding' (adhesion-based motility) and probably uses parasite-driven processes to gain entry into the cell initially.

Mele *et al.* (2004) have shown that depending on its developmental stage, *C. parvum* can inhibit (at the trophozoite stage) or promote (at the sporozoite and mezozoite stages) host cell apoptosis, indicating a parasite-driven regulation of host cell gene expression. Although the molecular mechanisms causing diarrhoea are largely unknown, infection of the epithelial surface results in loss of mature epithelial cells, causing shortening and fusion of the villi of the microvillus border. This leads to a decreased intestinal surface area, resulting in decreased absorption of fluids, nutrients and electrolytes as well as the loss of membrane-bound digestive enzymes (Chen *et al.*, 2002; Tzipori and Ward, 2002; Thompson *et al.*, 2005). According to Tzipori and Ward (2002), this loss of membrane-bound digestive enzymes has huge implications (especially in children where its role is crucial) as it contributes to marked maldigestion in addition to malabsorption.

Cell-mediated immunity appears to be the major component involved in the resolution of cryptosporidiosis and resistance to infection (Chen *et al.*, 2002; Leav *et al.*, 2002b). All humans are susceptible to infection although those with an impaired immune system are more vulnerable. A correlation between risk of

infection and a reduction in CD4+ T cells has been observed, with counts less than 150 cells/ml leading to the development of severe and life-threatening diarrhoea (Flanigan *et al.*, 1992; Leav *et al.*, 2002b; Riggs, 2002; Thompson *et al.*, 2005; Sunnotel *et al.*, 2006). Infection with *Cryptosporidium* elicits a local inflammatory response and increases the production of many cytokines, particularly interferon-gamma (IFN-gamma) (Chen *et al.*, 2002; Hommer *et al.*, 2003; Caccio and Pozio, 2006). According to Chen *et al.* (2002), IFN-gamma is important for resistance to *C. parvum* because absence of the cytokine in both gene knock-out mice and humans with IFN-gamma deficiency results in high levels of susceptibility to *C. parvum* infection. However, more extensive research is required to elucidate the human immune response to *Cryptosporidium* infection especially as most studies have concentrated on *C. parvum* infection and findings may not extend to other species (Riggs, 2002).

Although animal models, such as rodents, have been used extensively in attempting to explain the human immune response to *Cryptosporidium* infection, information from animal models is limited. For example, mice, in contrast to humans, do not suffer from diarrhoea post-infection (Pantenburg *et al.*, 2008). In addition, the immune response differs between mice and humans as IFN-gamma production seems to be associated with innate and primary immune responses in mice (Pantenburg *et al.*, 2008). Thus, rodents do not represent an optimal animal model for the study of human responses to *Cryptosporidium* infection. Non-human primates are probably the best *in vivo* model to mimic human infection but these models are both expensive and difficult to work with (Pantenburg *et al.*, 2008), hence data from animal models remain limited.

1.4.2 Species, genotypes and subtypes infecting humans

As previously mentioned there are currently 21 recognised species of *Cryptosporidium* (Table 1.2) and at least 8 of these are known to infect humans: *C. parvum, C. hominis, C. meleagridis, C. suis, C. felis, C. canis, C. muris C. andersoni* and possibly *C. baileyi*.

1.4.2.1 Cryptosporidium parvum

C. parvum was first described in mice by Tyzzer (1912), when he noted significant morphological and biological differences to *C. muris* [discovered by Tyzzer in 1907 (Tyzzer, 1907)]. It was initially thought that *C. parvum* infected over 150

mammalian hosts but many of these studies identified the presence of *C. parvum* using microscopy and so may not be as reliable as more recent studies which have used molecular techniques. For many years, this led to confusion about the host range of *C. parvum*. The development of molecular methods, such as DNA sequencing, indicated that *C. parvum* could be split into genotype I (human genotype now called *C. hominis*) and genotype II (bovine genotype now *C. parvum*), both infecting humans. This delineation of *C. parvum* into 2 separate species was confirmed by various genetic and cross transmission studies (Bonnin *et al.*, 1996; Peng *et al.*, 1997; Morgan *et al.*, 1998; Spano *et al.*, 1998; Widmer *et al.*, 1998; Homan *et al.*, 1999; Morgan *et al.*, 1999b; Xiao *et al.*, 1999b; Xiao *et al.*, 1999a; McLaughlin *et al.*, 2000; Morgan *et al.*, 2000a; Xiao *et al.*, 2000a; Alves *et al.*, 2001; Xiao *et al.*, 2001b; Morgan *et al.*, 2002).

The delineation of *C. hominis* and *C. parvum* was shown conclusively by McLaughlin *et al.* (2000) when 1705 *Cryptopsoridium* isolates from human faecal samples and 105 from livestock were analysed by PCR-restriction fragment length polymorphism (RFLP). Overall genotype I (*C. hominis*) was present in 37.8% of human samples with genotype II (*C. parvum*) present in 61.5% and *C. meleagridis* present in 0.3%. All livestock samples contained *C. parvum*. In addition to *C. hominis*, *C. canis* and *C. suis*, also once thought to be *C. parvum*, have since been elevated to species status (Xiao and Ryan, 2008). To date, *C. parvum* is believed to infect mainly ruminants (cattle, sheep, goats and deer) and humans (Xiao et al., 2004) though natural infections have been found in mice and raccoon dogs (Morgan *et al.*, 1999c; Matsubayashi *et al.*, 2004).

1.4.2.2 Cryptosporidium hominis

Once thought to be *C. parvum* genotype I, *C. hominis* is now considered a separate species based on both biological and molecular differences and was validated as a distinct species by Morgan *et al.* (2002). Extensive research has shown that *C. hominis* and *C. parvum* differ extensively; biologically in the location of infection [*C. parvum* found in the epithial cells of the intestine and *C. hominis* in the small bowel in humans (Hashim *et al.*, 2004)], genetically at a wide range of loci (Johnson *et al.*, 1995; Bonnin *et al.*, 1996; Peng *et al.*, 1997; Spano *et al.*, 1998; Widmer *et al.*, 1998; Homan *et al.*, 1999; Morgan *et al.*, 1999a; Morgan *et al.*, 1999b; Morgan *et al.*, 1999c; Xiao *et al.*, 1999b; Xiao *et al.*, 1999a; McLaughlin *et al.*, 2000; Morgan *et al.*, 2000a; Alves *et al.*, 2001; Xiao *et al.*, 2001a; Gatei *et al.*, 2002b; Chalmers *et al.*, 2005) and clinically in the symptoms of human infections (see section 1.4.4).

Cross-transmission studies have indicated that *C. hominis*, unlike *C. parvum*, has a very narrow host range. This species was considered non-infectious to mice, rats, cats, dogs and cattle (Xiao *et al.*, 2004). It was previously thought that *C. hominis* was limited to infections in human hosts, however, subsequent studies have found natural infections in a dugong (Morgan *et al.*, 2000c), cattle (Smith *et al.*, 2005b) and in a goat and a sheep (Giles *et al.*, 2009). *C. parvum* and *C. hominis* are the dominant species which infect humans worldwide.

1.4.2.3 Cryptosporidium meleagridis

C. meleagridis was first discovered in turkeys by Slavin (1955). The oocysts are morphologically indistinguishable from those of *C. parvum* (Xiao *et al.*, 2004) however, molecular analysis has confirmed its genetic uniqueness and coupled with biological studies, *C. meleagridis* species status was validated (Xiao *et al.*, 1999b; Morgan *et al.*, 2000b; Sréter *et al.*, 2000; Xiao *et al.*, 2000a; Glaberman *et al.*, 2001; Morgan *et al.*, 2001).

Initially, *C. meleagridis* was thought to infect only avian hosts but oocysts were transferred to immunosuppressed mice and from mice to chickens (Xiao *et al.*, 2004). Since then *C. meleagridis* has been identified in both immunosuppressed patients (Morgan *et al.*, 2000a; Guyot *et al.*, 2001; Gatei *et al.*, 2002b) and in immunocompetent patients (McLaughlin *et al.*, 2000; Pedraza-Diaz *et al.*, 2000; Xiao *et al.*, 2001a; Gatei *et al.*, 2006a; Cama *et al.*, 2007; Gatei *et al.*, 2007) and so is increasingly recognised as an important human pathogen. Although not found as commonly in humans as *C. hominis* and *C. parvum*, according to Xiao and Ryan (2008), *C. meleagridis* is the third most common infection, accounting for 10-20% of human cryptosporidiosis cases in Peru and Thailand (Xiao *et al.*, 2001a; Gatei *et al.*, 2002b; Cama *et al.*, 2003). In addition, it accounts for 0.6% of cases (19/3,100) in England and Wales (Chalmers *et al.*, 2002). Phylogenetic analysis of isolates places *C. meleagridis* in a clade with mammalian species and closely related to *C. parvum*, *C. hominis*, *C. wrairi* and other genotypes infecting primates, lagomorphs and humans (Xiao *et al.*, 2004). This indicates that *C. meleagridis* may have been originally a mammalian species which has now adapted to avian hosts.

1.4.2.4 Cryptosporidium canis

C. canis was first described in dogs in 1999 (Xiao *et al.*, 1999a) and was named the 'dog genotype'. It was validated as a separate species in 2001 (Fayer *et al.*,

2001) and was named *C. canis*. It is morphologically indistinguishable from *C. parvum* and oocysts have the ability to infect humans and bovines but not mice (Xiao *et al.*, 2004). *C. canis* was first described in humans by Pedraza-Diaz (2001) in patients in England. Subsequently, it has been isolated in humans in the USA, Thailand, Kenya, Peru, New Zealand and France (Pieniazek *et al.*, 1999; Guyot *et al.*, 2001; Xiao *et al.*, 2001a; Gatei *et al.*, 2002b; Tiangtip and Jongwutiwes, 2002; Cama *et al.*, 2003; Learmonth *et al.*, 2004; Gatei *et al.*, 2006b; Cama *et al.*, 2007). These studies incorporate both immunosuppressed and immunocompetant patients with and without diarrhoea. Confirmed *C. canis* infections have also been identified in dogs and foxes (Fayer *et al.*, 2001; Abe *et al.*, 2002; Zhou *et al.*, 2004).

1.4.2.5 Cryptosporidium felis

C. felis was first named in 1979 (Iseki, 1979) when it was identified in cats. Molecular characteristics have been used to validate it as a species with significant differences in DNA sequences from other species and genotypes (Xiao *et al.*, 2004). All *C. felis* isolates are consistent in various geographic regions (Morgan *et al.*, 1998; Morgan *et al.*, 1999c; Xiao *et al.*, 1999b). Originally the species was thought to be only infective to cats but since then studies have reported infections in cattle (Bornay-Llinares *et al.*, 1999) and both immunosuppressed and immunocompetant adults and children from various geographic areas including France, Italy, Portugal, Switzerland, UK, USA, India, Kenya, Peru and Thailand (Pieniazek *et al.*, 1999; Morgan *et al.*, 2000a; Alves *et al.*, 2001; Guyot *et al.*, 2001; Pedraza-Diaz *et al.*, 2001; Caccio *et al.*, 2002; Gatei *et al.*, 2002b; Tiangtip and Jongwutiwes, 2002; Matos *et al.*, 2004; Muthusamy *et al.*, 2006; Ajjampur *et al.*, 2007; Cama *et al.*, 2007; Gatei *et al.*, 2007).

1.4.2.6 Cryptosporidium muris

C. muris was the first species of *Cryptosporidium* identified by Tyzzer in 1907 in the gastric epithelium of mice (Tyzzer, 1907). Phlylogenetic analysis of the small subunit (SSU) rRNA sequence confirmed *C. muris* as a distinct species (Xiao *et al.*, 1999a). Initially many studies were carried out on morphological data without genetic confirmation and so results may be putative. These studies found *C. muris* (or '*C. muris*-like') oocysts in cattle in various geographic locations including Brazil, USA, Iran and Scotland and natural infections have been reported in more recent studies in mice, cattle, bilbies, squirrels, chipmunks, hamsters, woodmice, bank voles, rock hyrax, mountain goats, cynomolgus monkeys and camels (Xiao

et al., 2004). There have been several confirmed *C. muris* infections in immunosuppressed humans in Kenya, Peru, Thailand and India and a putative cases in France (Guyot *et al.*, 2001; Gatei *et al.*, 2002a; Tiangtip and Jongwutiwes, 2002; Gatei *et al.*, 2003; Palmer *et al.*, 2003; Gatei *et al.*, 2006a; Muthusamy *et al.*, 2006). In addition, a putative case of *C. muris* infection in 2 healthy Indian girls was reported but there was no molecular evidence for confirmation (Katsumata *et al.*, 2000).

1.4.2.7 Cryptosporidium suis

C. suis, previously known as the 'pig genotype', was first identified in pigs and determined a valid species in 2004 (Ryan *et al.*, 2004) following molecular and phylogenetic analysis (oocysts are indistinguishable from *C. parvum*) (Morgan *et al.*, 1998; Morgan *et al.*, 1999a; Enemark *et al.*, 2003; Ryan *et al.*, 2003; Ryan *et al.*, 2004). It has been identified in pigs, a calf and a lamb (Morgan *et al.*, 1998; Enemark *et al.*, 2003; Fayer *et al.*, 2006; Ryan *et al.*, 2008) and also in humans. An isolate of *C. suis* was identified in HIV-positive patients in Lima, Peru (Xiao *et al.*, 2001a; Cama *et al.*, 2003; Cama *et al.*, 2007) and in a patient in England (Leoni *et al.*, 2006).

1.4.2.8 Cryptosporidium andersoni

C. andersoni was first described by Anderson in 1987 (Xiao *et al.*, 2004) and validated by Lindsey in 2000 (Lindsay *et al.*, 2000) following genetic, cross transmission and biological studies (Lindsay *et al.*, 2000; Sréter *et al.*, 2000; Hijjawi *et al.*, 2002). Due to the similarities between the oocysts of *C. muris* and *C. andersoni*, studies using microscopy alone have resulted in confusion over whether the isolates discovered in certain hosts or used in cross-transmission studies were *C. muris* or *C. andersoni*. Studies have described infections in cattle (Wade *et al.*, 2000; Enemark *et al.*, 2003; Peng *et al.*, 2003a; Santin *et al.*, 2004) and natural infections have occurred in a sheep and bactrian camel (Xiao *et al.*, 2004; Wang *et al.*, 2008). *C. andersoni* has been found to be non-infectious in mice, however in Japan a novel type of *C. andersoni* has been put forward based on its ability to infect immunodeficient mice (Satoh *et al.*, 2003).

There has been only 1 confirmed case of *C. andersoni* in humans using molecular analysis. Leoni *et al.* (2006) identified a *Cryptosporidium* oocyst in a patient with diarrhoea in England which was more similar to *C. andersoni* (99% identity) than to *C. muris* (98% identity). *C. andersoni*-like oocysts were observed in 3 patients

in England and in a child in Malawi (Lindsay *et al.*, 2000; Morse *et al.*, 2007). In addition, a putative *C. muris* infection was reported in an immunosuppressed patient in France but the sequence was more similar to that of *C. andersoni* than *C. muris* (Guyot *et al.*, 2001)

1.4.2.9 Cryptosporidium baileyi

C. baileyi was first described in broiler chickens (Xiao *et al.*, 2004) and was considered a separate species due to its larger size in comparison to *C. meleagridis* and its development in the respiratory tract. It is generally considered to be an avian parasite found naturally in chickens, gulls, quail, falcons and cockatiels (Current *et al.*, 1986; Pavlasek, 1993; Morgan *et al.*, 2001; Abe and Iseki, 2004; Hajdusek *et al.*, 2004; van Zeeland *et al.*, 2008). *C. baileyi* was apparently discovered in an immunosuppressed human in 1991 (Ditrich *et al.*, 1991). In autopsy material, the parasite was found in the oesophagus, whole intestine, trachea, larynx, lungs and urinary and gall bladders. Cross-transmission in suckling mice showed no infectivity but 26 chickens did become infected (Ditrich *et al.*, 1991). However, there have been no subsequent reports of infections with *C. baileyi* in humans and according to Hunter and Nichols (2002) the organism isolated from this immunosuppressed human was later shown to be antigenically different from *C. baileyi*.

1.4.2.10 Genotypes infecting humans

Over 40 genotypes of *Cryptosporidium* have been described owing to uncertainty associated with taxonomy. According to Xiao and Ryan (2008), genotypes are named after substantial sequence differences (greater than or comparable to those between established genotypes that become species) in the SSU rRNA or other genes are found and phylogenetic analysis has eliminated the possibility that differences are due to heterogeneity between copies of the rRNA gene. Not all genotypes differ from each other to the same extent. As discussed by Xiao and Ryan (2008), a genotype is not a taxon, but a 'partial and temporary descriptor which highlights the incompleteness of our knowledge of the parasite'. With further work genotypes are likely to become valid species as was the case for *C. suis* and *C. canis.*

At least 7 genotypes are known to infect humans - Chipmunk genotype I, *C. hominis* monkey genotype, skunk, rabbit, horse, pig genotype II and most commonly the cervine genotype. The cervine genotype was initially found in storm

water in China (Xiao *et al.*, 2002) and since then has been found to infect domestic and wild ruminants, rodents and primates (lemurs and humans) (Xiao and Feng, 2008). It was first reported in humans by Ong *et al.* (2002) in 9 patients in British Columbia. Since then it has been reported sporadically from individuals in Ontario, the USA, Slovenia and England (Feltus *et al.*, 2006; Leoni *et al.*, 2006; Trotz-Williams *et al.*, 2006; Soba and Logar, 2008).

The 6 remaining genotypes account for very few human infections. The chipmunk genotype was found in 2 patients in Wisconsin (Feltus *et al.*, 2006), *C. hominis* monkey genotype in 2 patients in the UK (Mallon *et al.*, 2003), the pig genotype II in an immunocompetent man in the Czech Republic (Kvac *et al.*, 2009) and the rabbit, horse and skunk genotypes in immunocompetent women in England (Robinson *et al.*, 2008b). The rabbit genotype was also responsible for a waterborne outbreak in the UK in 2008 (Chalmers *et al.*, 2009a).

1.4.2.11 Subtypes of Cryptosporidium

Subtypes have relatively minor intragenotypic variations in *Cryptosporidium* species especially for *C. parvum* and *C. hominis* (Xiao and Ryan, 2008). Various tools for subtyping these species have been developed, including glycoprotein 60 (GP60) sequencing (Strong *et al.*, 2000; Glaberman *et al.*, 2001; Glaberman *et al.*, 2002; Leav *et al.*, 2002a; Peng *et al.*, 2003a) and sequencing of minisatellite (MN) and microsatellite (MS) markers (Caccio *et al.*, 2000; Enemark *et al.*, 2002; Mallon *et al.*, 2003). Micro- and mini- satellites are DNA sequences that consist of tandemly repeated sequence motifs 1-4 bp (micro) or more (mini) and the genetic variations in micro– and mini- satellites are generally variations in the number of these tandem repeats (Xiao and Ryan, 2008).

Similarly, the GP60 gene contains tandem repeats of the serine—coding trinucleotide region. GP60 encodes a 60-kilodalton (kDa) precursor glycoprotein (GP) that is cleaved to yield 40kDa GP40 and 15kDa GP15 sporozoite surface glycoproteins (Akiyoshi *et al.*, 2006). GP40 binds specifically to host cells, and anti-bodies against this protein neutralises *Cryptosporidium* infections *in vitro* suggesting that this surface protein is involved in parasite adhesion and invasion (Zhu, 2008).

GP60 has been identified as the most polymorphic gene to date and several subtype allelic families have been described for both *C. hominis* and *C. parvum*. These subtype families are named based upon the ~900bp DNA sequence

downstream of the microsatellite region of the GP60 gene. *C. parvum* subtype alleles of the family II have been described as IIa, IIb, IIc, IId, IIe, IIf, IIg, IIh (Xiao and Ryan, 2008) and more recently IIi in children in Uganda (Akiyoshi *et al.*, 2006), IIj in cattle in Northern Ireland (Thompson *et al.*, 2007), IIk in a racoon dog in Japan (Abe *et al.*, 2006), IIl in humans in Slovenia as well as in cattle in Slovenia, Serbia, Montenegro and The Netherlands (Misic and Abe, 2007; Soba and Logar, 2008; Wielinga *et al.*, 2008) and IIm in children from Bangladesh (Hira *et al.*, Unpublished, Accession No. AY700401) have been identified. IIa and IIc are by far the most commonly detected worldwide, IIc in humans and IIa in both cattle and humans. Likewise *C. hominis* subtype alleles of the family I have been described as Ia, Ib, Id, Ie, If, Ig (Xiao and Ryan, 2008).

Within each subtype family, tandem repeats of serine-coding trinucleotides [TCA (coded by the letter A) TCG (coded by the letter G) and TCT (coded by the letter T) at the 5′ end of the gene (Xiao and Ryan, 2008)] have been identified and most subtype families contain multiple subtypes that differ primarily by the length and type of serine repeats (Akiyoshi *et al.*, 2006) (Table 1.4). Repeat regions can also occur in subtypes Ia and IIa and are given the code R1 (1 repeat), R2 (2 repeats) etc. Subtypes of *C. parvum* and *C. hominis* at the GP60 locus are named based on their subtype family designations, the number of each type of trinucleotide repeats and whether or not there is a repeat region present (Sulaiman *et al.*, 2005).

Table 1.4 Major GP60 subtype families [modified from Xiao and Ryan, (2008)]

Species	Subtype Family	Dominant trinucleotide repeat	Other repeat (R)
C. hominis	Ia	TCA	AAAA/GCGGTGGTAAGG
	Ib	TCA, TCG, TCT	
	Id	TCA, TCG	
	Ie	TCA, TCG, TCT	
	If	TCA, TCG	
	Ig	TCA	
C. parvum	IIa	TCA, TCG	ACATCA
	IIb	TCA	
	IIc	TCA, TCG	
	IId	TCA, TCG	
	IIe	TCA, TCG	
	IIf	TCA	
	IIg	TCA	
	IIh	TCA, TCG	
	IIi	TCA	
	IIj	TCA, TCG	ACATCA
	IIk	TCA	ACATCA
	IIl	TCA	ACATCA
	IIm	TCA, TCG	

Subtyping tools may be useful to distinguish zoonotic species from anthroponotic species to a higher degree of specificity than genotyping. For example, it was originally believed that *C. parvum* infection indicated a zoonotic infection with the potential for anthroponotic transmission once infected. However, using GP60 subtyping analysis evidence from the USA, Portugal, Peru and South Africa has indicated that *C. parvum* of the allele family IIc has to date not been identified in animals (Leav *et al.*, 2002a; Alves *et al.*, 2003; Xiao *et al.*, 2004; Xiao and Ryan, 2004; Alves *et al.*, 2006; Areeshi *et al.*, 2008). In contrast, the *C. parvum* subtype IIa has been identified in both cattle and humans (Leav *et al.*, 2002a; Peng *et al.*, 2003b; Xiao *et al.*, 2004). Similarly, multilocus genotyping of *C. parvum* isolates from humans and animals in Scotland using micro- and mini-satellite markers (Mallon *et al.*, 2003) identified 2 subtypes that were detected in

humans but not in sheep or cattle. These studies suggest that either these *C. parvum* isolates are specific to humans, or researchers have yet to detect these genotypes in animals.

Several subtypes of *C. meleagridis* have also been described based on mulltilocus analysis (Glaberman *et al.*, 2001). Of the 11 human and bird isolates characterised, 6 subtypes have been seen in the HSP70 gene, 6 in the GP60 gene (indicating high heterogeneity in these genes) and 2 subtypes were identified in the SSU rRNA gene (Glaberman *et al.*, 2001). Detailed information, obtained through subtype analysis of *C. meleagridis* (and likewise for *C. hominis* and *C. parvum*), will enable researchers to define more clearly the epidemiology of these species and highlight the public health significance of the parasite.

1.4.3 Infectious dose

Cryptosporidium is highly infectious with an ID_{50} (infectious dose to 50% of exposed individuals) ranging from 9-1042 oocysts (Okhuysen *et al.*, 1999). Okhuysen *et al.* (1999) observed heterogeneity in infectivity of isolates of *Cryptosporidium*. Three distinct isolates of *C. parvum*, IOWA, UCP and TAMU had ID_{50} s of 87 (CI, 48-126), 1042 (CI, 0-3004) and 9 (CI, 4-14) respectively. DuPont *et al.* (1995) infected 29 healthy volunteers with IOWA *C. parvum* and determined the ID_{50} to be 132 oocysts.

Prior exposure to *C. parvum* oocysts was demonstrated to provide protection from infection and illness at low oocyst doses (Chappell *et al.*, 1999). The ID_{50} was 1880 oocysts in previously seropositive patients, 20 times greater than in seronegative volunteers, thus, the infectious dose appears to depend not only on the strain of parasite but also on the immune status of the host.

1.4.4 Clinical symptoms

Cryptosporidium causes the disease cryptosporidiosis which is characterised by acute watery diarrhoea, malabsorption and wasting. Intensity and duration of disease will depend upon a combination of host (age, immune status, nutritional status, previous exposure and ID_{50}) and parasite (origin, age of oocysts and species/genotype) factors (Tzipori and Ward, 2002).

In immunocompetent patients, cryptosporidiosis is acute and self-limiting. However, it can be much more severe in patients with a compromised immune system (especially those with HIV/AIDS and the malnourished). It can lead to chronic dehydration and even to death (Dillingham *et al.*, 2002). Watery diarrhoea is the primary symptom of infection with less frequent symptoms including crampy abdominal pains, nausea, malaise, vomiting, low grade (<39°C) fever and loss of appetite (Fayer and Ungar, 1986; Jokipii and Jokipii, 1986; Leav *et al.*, 2002b). In addition, extra-intestinal symptoms such as decreased mental development may occur (Guerrant *et al.*, 1999). Symptoms begin approximately 7 days after ingestion of oocysts (Jokipii and Jokipii, 1986; Tzipori and Ward, 2002; Chappell *et al.*, 2006) and the duration of diarrhoea in immunocompetent individuals can vary from several days to 5 weeks (Jokipii and Jokipii, 1986; Leav *et al.*, 2002b; Chappell *et al.*, 2006). In immunocompromised patients, however, the illness can last for months or even years. Infection can be extremely severe in HIV-positive patients with passage of >2L/day or 'cholera-like' diarrhoea (Fayer, 2008). In these patients, infection can often spread from the gut to hepatobiliary and pancreatic ducts causing cholangiohepatitis, cholecystitis, choledochitis or pancreatitis (Tzipori and Ward, 2002). It has been indicated that different species, and even subtypes, can cause distinct clinical symptoms.

Differences in oocyst shedding patterns and intensity of infection between *C. hominis* and *C. parvum* have been documented. McLaughlin *et al.* (1999) found that patients infected with *C. hominis* shed significantly more oocysts in comparison with *C. parvum* infections. Similar results were observed by Xiao *et al.* (2001a) and Bushen *et al.* (2007) when it was found that the duration of oocyst shedding and intensity of infection was higher for patients infected with *C. hominis* than *C. parvum*. In addition, Bushen *et al.* (2007) indicated the height-for-age z-scores showed a significant decrease within 3 months of infection with either *C. hominis* or *C. parvum*. However, 3-6 months following infection only *C. hominis*-infected children continued to show a decrease in their height-for-age z-scores. Cama *et al.* (2008) indicated that distinct genotypes and even subtypes were linked to different clinical manifestations. *C. hominis* was associated with increased oocyst shedding, intensity and duration while in contrast, *C. parvum*, *C. meleagridis, C. canis* and *C. felis* were associated with diarrhoea only. All *C. hominis* subtypes isolated were associated with diarrhoea (Ia, Ib, Id and Ie), and Ib was also associated with nausea, vomiting and general malaise. This indicates that *C. hominis* subtype Ib may be more pathogenic than other subtypes (Cama *et al.*, 2008).

1.4.5 Treatment

Over the past 2 decades, many studies have been carried out on possible treatments for cryptosporidiosis and although much information has been gathered for various drugs, as of yet there are no consistently effective, approved products for either animals or humans (Tzipori and Ward, 2002; Fayer, 2004; Thompson *et al.*, 2005). The lack of available treatment is mainly owing to a poor understanding of host-parasite interactions, in addition to the inability, until recently, to culture the parasite *in vitro* in cell-free media. However, with the development of genome sequencing, molecular genotyping and the relatively recent description of culturing *C. parvum* in cell-free media (Hijjawi *et al.*, 2004), the development of effective drugs is increasingly likely. According to Tzipori and Ward (2002), the identification of key molecules, such as metabolic enzymes or surface proteins that mediate parasite attachment and invasion, are likely to be exploited as potential targets for chemotherapy.

Oral rehydration therapy (ORT) is commonly used in the treatment of cryptosporidiosis in order to diminish clinical signs of illness in humans. ORT involves the oral consumption of fluids, electrolytes and salts to replenish those lost due to diarrhoea. This form of treatment is administered until the patient develops sufficient immunity to clear the infection (Thompson *et al.*, 2005). However, ORT only treats the symptoms and not the cause of infection and therefore, may not be effective for those with an impaired immune system.

According to Ramirez *et al.* (2004) over 150 antimicrobial drugs have been studied for the treatment of cryptosporidiosis with only a small number showing a decrease in disease but none eliminating the infection completely. In 2002, the US Food and Drug Administration approved the drug nitazoxanide (Alinia) for the treatment of paediatric diarrhoea by *C. parvum* in children aged 1-11 years (Ramirez *et al.*, 2004). In 2005, this drug was approved for adults and teenagers with cryptosporidiosis (Fox and Saravolatz, 2005). Nitazoxanide (NTZ), a nitrothiazole benzamide, is a broad spectrum antimicrobial agent with studies showing activity against protozoa, nematodes, cestodes, trematodes and bacteria (Ortiz, 2001; Amadi *et al.*, 2002; Gilles and Hoffman, 2002; Rossignol, 2006; Rossignol *et al.*, 2006). A number of human clinical trials have been carried out to determine the effectiveness of NTZ in treating cryptosporidiosis (Doumbo *et al.*, 1997; Rossignol *et al.*, 1998; Rossignol *et al.*, 2001). Results indicated that NTZ treatment decreases the severity of diarrhoea and reduces oocyst shedding although it may not clear the infection entirely.

Recently, Rossignol *et al.* (2006) have shown that a 3-day course of NTZ was effective in treating diarrhoea and enteritis caused by *Cryptosporidium* in non-immunodeficient patients over the age of 12 years. In a subsequent study, 357 patients suffering from AIDS-related cryptosporidiosis in the USA were treated with 500-1500mg of NTZ twice a day (Rossignol, 2006). The study showed that 59% of the patients achieved substantial clinical response while on the treatment and the author indicated that NTZ is a useful therapy for the treatment of AIDS-related cryptosporidiosis. In contrast, an earlier study found that a 3-day course of NTZ significantly improved the resolution of diarrhoea, parasitological eradication and mortality in HIV-negative but not HIV-positive children (Amadi *et al.*, 2002).

Giacometti *et al.* (2000) tested the *in vitro* activity of NTZ alone and in combination with azithromycin and rifabutin against 4 clinical isolates of *C. parvum*. Results found that no product produced complete inhibition of parasite growth but NTZ exhibited the highest anticryptosporidial activity and suppressed growth of meronts and gamonts by 51.6%. When the 3 drugs were combined, there was a parasite reduction of 79.8-83.9%.

Meta-analysis was carried out on 7 trials involving 169 immunocompromised individuals with cryptosporidiosis and treated with NTZ and paromomycin and found no evidence of effectiveness for the reduction in duration of diarrhoea for either drug (Abubakar *et al.*, 2007). NTZ led to a significant increase in the clearance of oocysts compared with placebo (relative risk: 0.52, CI: 0.3-0.91) but the effect was not significant for seropositive participants. This research indicates that there is little evidence for the efficacy of NTZ for the treatment of cryptosporidiosis.

In summary, combined research to date suggests that NTZ may play a part in reducing clinical symptoms and oocyst shedding in immunocompetant patients, but in immunocompromised patients it is unlikely that NTZ will clear the infection completely.

Paromomycin (an aminoglycoside antibiotic) is one of the most widely used drugs to treat cryptosporidial infection in AIDS patients. The drug is poorly absorbed in the gastrointestinal tract but, according to Thompson *et al.* (2005), small amounts cross the apical membrane surrounding the parasite and may act against extra- and intra-cellular parasites. The drug has been shown to decrease clinical

symptoms and oocyst shedding in mice, calves and chickens (Fayer and Ellis, 1993; Blagburn *et al.*, 1998; Sréter *et al.*, 2002).

In human trials on paromomycin, there are varying results with noted decreases in oocyst shedding and stool frequency. However, many patients continue to shed oocysts and others have relapsed during treatment. Danziger *et al.* (1993) reported a case of 1 AIDS patient with cryptosporidial diarrhoea successfully treated with paromomycin. However, diarrhoea reoccurred when therapy was discontinued. Similarly, Blanshard *et al.* (1997) tested the safety and efficacy of paromomycin in patients with AIDS and cryptosporidiosis. The treatment resulted in complete resolution of diarrhoea in 60% of patients but did not eliminate the infection completely. White *et al.* (1994) set up a double-blind, placebo-controlled study. Ten patients with AIDS and cryptosporidiosis were randomised to paromomycin or placebo for 14 days and then switched to the other treatment for 14 days. While taking paromomycin, patients exhibited decreased oocyst shedding, suggesting clinical improvement. However, parasite load remained high in some patients and infection was not cleared entirely. Giacometti *et al.* (1999) treated a HIV-positive woman with cryptosporidiosis with paromomycin. Only slight improvements in clinical symptoms were observed. Likewise, Hewitt *et al.* (2000) set up a prospective double-blind, placebo-controlled trial and treated 17 immunocompromised adults with paromomycin and 18 adults with placebo for 21 days. Three treatment (17.6%) compared with 2 placebo (14.3%) patients responded completely. It was concluded that paromomycin is no more effective than placebo for the treatment of cryptosporidial diarrhoea. However, the statistical power of the analysis was low and so the efficacy of the drug cannot be fully rejected. Although paromomycin is widely used to treat patients with cryptosporidiosis, it would appear that treatment is problematic and rarely clears the infection entirely.

Several other drugs including clarithromycine (Jordan, 1996; Holmberg *et al.*, 1998; Fichtenbaum *et al.*, 2000) azithromycine (Blanshard *et al.*, 1997; Holmberg *et al.*, 1998; Giacometti *et al.*, 1999; Trad *et al.*, 2003), roxithromycine (Uipa *et al.*, 1998), diclazuril and letrazuril (Blanshard *et al.*, 1997; Mead, 2002), and rifabutin (Holmberg *et al.*, 1998; Fichtenbaum *et al.*, 2000; Giacometti *et al.*, 2000) have been studied with varying results.

Hyperimmune bovine colostrums (HBC), high in anti-cryptosporidial antibodies, has been tested for efficiency in mice, calves and humans with mixed results (Fayer, 2004). HBC is produced by vaccinating cows during pregnancy and

collecting postnatal colostrums rich in immunoglobulins (primarily IgG) (Thompson *et al.*, 2005). Greenberg and Cello (1996) used HBC to treat AIDS patients diagnosed with cryptosporidiosis and found that there was a decrease in mean stool weight and stool frequency, however the effect on parasite load was not tested. Okhuysen *et al.* (1998) investigated the prophylactic effect of bovine hyperimmune anti-*Cryptosporidium* (BACI) in healthy adults before and after being challenged with *C. parvum*. The results showed a trend toward less diarrhoea in subjects receiving BACI in comparison with controls but the results were not statistically significant ($p=0.08$); however, a 100-fold reduction in the number of oocysts excreted was observed in those adults receiving colostrums. Overall, the use of HBC tends to lead to a reduction in symptoms rather than complete clearance of the infection. In addition, probiotics such as *Lactobacillus reuteri, L. acidophilius* and *Bifidobacterium sp.* have been shown to decrease duration and number of oocysts shed in experimentally-infected animals and humans (Alak *et al.*, 1997; Alak *et al.*, 1999; Rotkiewicz *et al.*, 2001; Pickerd and Tuthill, 2004).

In relation to the immune system, cell-mediated immunity appears to be the major component in fighting *Cryptosporidium* infection. A correlation has been documented between a decreased number of CD4+ T cells and the risk of acquiring infection (Hunter and Nichols, 2002; Riggs, 2002). Huang *et al.* (2004) states that the most effective treatment for cryptosporidiosis for HIV-positive individuals is the use of highly active antiretroviral treatment (HAART) with infection decreasing as the number of CD4+ T cells increase. HAART increases CD4+ T cell counts and inhibits viral replication using nucleoside and non-nucleoside reverse transcription inhibitors and HIV protease inhibitors (Caccio and Pozio, 2006). Studies have also suggested that HAART is protective independently of CD4+ T cells and works by stimulating the production of IFN-gamma or by inhibiting parasitic proteases or both (Lean *et al.*, 2002; Riggs, 2002; Hommer *et al.*, 2003; Caccio and Pozio, 2006). In immunodeficient patients, it has been shown that intestinal infections can cause antiretroviral drug malabsorption in patients with chronic diarrhoea and wasting (Brantley *et al.*, 2003). Bushen *et al.* (2004) demonstrated that antiretroviral drug levels increased in AIDS patients who were given alanyl glutamine or glutamine and predicted that these supplements may help to improve therapy for patients with AIDS who present with diarrhoea and/or wasting. However, the study has several limitations including the small number of patients enrolled (25).

The introduction of HAART has reduced the incidence of life-threatening cryptosporidiosis in AIDS patients in most developed countries (Tzipori and Ward, 2002). Unfortunately, in developing countries where AIDS is endemic, HARRT less widely available.

1.4.6 Prevention/control

Cryptosporidium infection is difficult to control owing to several characteristics including: the hardy, chlorine-resistant oocyst, the small size of the oocyst (problems with filtration), its low infectious dose (ID_{50}) ranging from 9 to 1042 oocysts depending on the isolate (Okhuysen *et al.*, 1999) [although it has been suggested that just 1 oocyst may cause infection (Eisenberg *et al.*, 1998)]; and the fact that the oocyst is infectious when shed and its zoonotic potential (Dillingham *et al.*, 2002).

Contaminated water is a major source of human infection and treatment of water with chlorine has been shown to be insufficient (Korich *et al.*, 1990; Pereira *et al.*, 2008). As discussed in Section 1.3.3, oocysts subjected to high and low temperatures, desiccation, salinity and pH can have varying effects on survival. Alternative methods for controlling the spread of oocysts in water include filtration, ultraviolet light inactivation and ozone treatment.

According to Bowman (2008), conventional filtration consisting of coagulation, sedimentation and filtration is capable of attaining at least 99.9% removal of *Cryptosporidium* oocysts. However, filtration alone requires a filter with a pore size of <1µm (Steiner *et al.*, 1997; Leav *et al.*, 2002b) and even in cases where water was treated under the conventional water treatment conditions, outbreaks have occurred (Mackenzie *et al.*, 1994).

The infectivity of *C. parvum* oocysts was tested in mice following exposure to ozone (an unstable oxidant) (Korich *et al.*, 1990) with greater than 90% inactivation achieved following the treatment of oocysts with 1ppm ozone for 5 minutes. Similarly, ozone treatment was determined more effective at rendering oocysts inactive (100% inactivity with a concentration of 24mg/L) than chlorine and chlorine dioxide (Pereira *et al.*, 2008) indicating that ozone may be practical for the treatment of water. However, although ozone can be used for the inactivation of oocysts, according to Clancy and Hargy (2008) it may not be as

effective as initially thought owing to reduced inactivation levels in water at low temperatures.

UV light has been shown to be an effective method for treatment of water. Inactivation of oocysts using UV light is a physical process. UV light damages an organism when molecules within the organism absorb photons of germicidal wavelength (Bowman, 2008). UV light and solar light have been shown to be effective when used on tap water (Drescher *et al.*, 2001; King *et al.*, 2008). King *et al.* (2008) carried out a study assessing natural sunlight and the inactivation of *C. parvum* oocysts. Results indicated that inactivation of up to 90% of oocysts occurred in the first hour in tap water but increased dissolved organic carbon content in environmental waters decreased solar inactivation. UV was also observed to be effective in inactivating 5 strains of *C. parvum* with all 5 strains being equally sensitive (Clancy *et al.*, 2004; Rochelle *et al.*, 2004).

Preventative measures are by far the most effective way to control the spread of *Cryptosporidium*. In addition to the use of physical processes in the treatment of water, good hygiene practices are vital in limiting infection. Good hygiene practices include extensive hand washing, avoiding contact with stools from animals and humans, avoiding accidental ingestion of water used in recreational activities (e.g. swimming pools) and improving both design and operation of water treatment facilities (Leav *et al.*, 2002b). Counselling of individuals regarding boiling and freezing may prove effective in preventing transmission of *Cryptosporidium*, however, according to Kosek *et al.* (2001), many individuals who were interviewed after a *Cryptosporidium* outbreak in the UK, who believed they were complying with the boiled water advisory, rinsed fresh food and brushed their teeth in un-boiled tap water. This leads to questions regarding the effectiveness of counselling individuals.

1.5 Diagnosis

Prior to 1980, the diagnosis of cryptosporidiosis was primarily histological. Parasitic life-stages were identified in the intestinal mucosa using biopsies and staining with haematoxylin and eosin stains. However, this procedure was invasive, expensive and time consuming, and the lack of effective diagnostic

techniques hindered progress in the recognition of *Cryptosporidium* as an important human pathogen (Smith, 2008). More conventional methods are now based on the use of microscopy coupled with various staining and concentration procedures. In recent years, with the development of molecular techniques, the species of *Cryptosporidium*, and even the genotypes and subtypes causing the infection can now be confirmed. This major advance in the diagnosis of *Cryptosporidium* infection has great implications for understanding the transmission dynamics and the public health impact of the parasite.

As stool samples are the primary specimens used for diagnosis, conventional techniques for identification of oocysts in human faeces will be briefly discussed, in addition to the more recently developed molecular techniques.

1.5.1 Oocyst concentration and separation from human faeces

If a patient is heavily infected with *Cryptosporidium* and a large number of oocysts are excreted in the stool, then a direct smear can be prepared. Smears are left to air-dry and are then stained. However, in samples with a low number of oocysts, sensitivity of diagnosis can be increased by concentrating the sample, applying it to a slide and staining. Concentration techniques partially purify and separate oocysts from debris within the stool sample and can also be used to increase the availability of nucleic acid for molecular work and decrease PCR inhibitors such as salts and heavy metals (Smith and Nichols, 2009b).

Concentration procedures include formal-ether concentration, sucrose flotation and zinc sulphate flotation. Water-ether concentration can be used when further molecular work is required. Formal-ether concentration uses formalin for fixation and preservation and ether to remove fats and oils. Centrifugation is then applied to enable oocysts in the sample to settle rapidly to the bottom. Oocyst flotation involves a liquid suspension which is denser than the oocysts, so that the oocysts rise to the top and can then be removed. Sucrose and zinc sulphate are used owing to their density and also to the fact that these solutions have little effect on oocyst viability.

Bukhari and Smith (1995) tested the effect of 3 concentration techniques - water-ether concentration, sucrose flotation and zinc sulphate flotation – on oocyst recovery and viability, taken from bovine faeces. Recovery was significantly higher for water-ether concentration and this technique did not exert a significant effect

on viability. Similar studies indicated that formal-ether concentration was superior to both sucrose and zinc sulphate flotation (Weber *et al.*, 1991; Mtambo *et al.*, 1992). According to Smith (2008) sucrose flotation is used rarely on human stools owing to the fat content and the fact that if oocysts are not thoroughly washed, sucrose can interfere with staining and adherence to microscopic slides. The modified formal-ether method, reported to be more sensitive than the method developed by Allen and Ridley (1970) (Casemore *et al.*, 1985) is recommended for stools with low numbers of oocysts (Smith, 2008).

Stool consistency can vary depending on a number of factors such as age, diet and disease status, with oocysts more easily recovered from liquid stools than solid or semi-solid stools, probably owing to the reduction in debris present (Bukhari and Smith, 1995; Nichols *et al.*, 2006b).

Immunomagnetic separation (IMS) is also used for concentration of oocysts and involves the binding of surface-exposed oocyst epitopes to magnetised beads. The beads are coated with monoclonal antibodies (mAbs) which recognise the oocyst's wall protein. Once bound, the bead-oocyst complexes are attracted to one side of a test tube using a permanent magnet. The oocysts are then recovered and dissociated from the beads in acidic solution (Campbell and Smith, 1997; Smith, 2008; Smith and Nichols, 2009b). According to Smith and Nichols (2009b), high turbidity, low pH and particulate matter can decrease the efficiency of IMS. Nichols *et al.* (2006b) used IMS to recover oocysts from seeded faecal samples and found that efficacy varied according to consistency: liquid (49.8 ± 8%, n=5), semisolid (43.2 ± 6.4%, n=5) and solid (29.5 ± 4.5%, n=5).

1.5.2 Staining

Due to the difficulty of observing colourless oocysts using light microscopy, acid-fast staining was initially adopted. The most commonly used acid-fast stains include modified Ziehl Neelson (mZN) (Casemore, 1991) and Kinyoun stain (Kehl *et al.*, 1995) in conjunction with negative staining using carbol fushin. However, limitations include poor detection rates and misdiagnosis due to the staining of spores which may be misinterpreted.

Today, a number of fluorogenic stains are used and tend to be more sensitive than acid-fast stains. Auramine-phenol (AP) coupled with negative staining with carbol fushin or potassium permanganate is used widely (Smith, 2008) and,

according to Casemore *et al.* (1985), is more sensitive than mZN as both the outer wall and the internal structures are stained. Oocysts and sporozoites can be seen with the fluorescein isothiocyanate (FITC) filter and UV filters of an epifluorescent microscope. In addition, Morse *et al.* (2007) showed that AP staining was more senitive than both mZN staining and immunoflourescence (IF). Using AP stain 40/50 positive samples were identified, however, IF identified only 36/50 samples and mZN 31/50 samples (Morse *et al.*, 2007). However, limitations to AP staining and microscopy arise due to the non-specific staining of yeasts and other debris that may be mistaken for oocysts, particularly with mZN. AP is recommended for stools with very few oocysts (Smith, 2008).

1.5.3 Immunological assays

Immunological-based techniques include enzyme immunoassays (EIA), immunofluorescence with monoclonanl antibodies (mAbs) and enzyme-linked immunosorbent assays (ELISA). Immunofluorescent monoclonal antibodies recognise the surface-exposed epitopes of oocysts and can be more sensitive than conventional staining methods (Garcia *et al.*, 1987; Arrowood *et al.*, 1991; Weber *et al.*, 1991). As with fluorescent stains, this method requires the use of an epifluorescent microscope. mAbs are genus-specific and the binding of antibody paratopes in FITC-C-mAbs to epitopes results in fluorescence, enabling improved morphometric analysis (Smith, 2008). The use of mAbs may be enhanced by using flourgen 4' 6-diamidino-2-phenyl indole (DAPI) (Grimason *et al.*, 1994; Smith *et al.*, 2002; Smith *et al.*, 2003). DAPI causes the nuclei of sporozoites to fluoresce blue using a UV filter on a fluorescent microscope. This allows the integrity of the nuclei to be assessed and can also be useful prior to PCR to determine the presence or absence of nuclei particularly with low oocysts numbers (Smith and Nichols, 2009b). In a study conducted by Arrowood *et al.* (1991), it was found that oocysts in infected tissues were easily detected by mAb-based methods which were superior to both acid-fast stain or auramine-rhodamine staining. Similar results were observed by Garcia *et al.* (1987). However, the equipment used to carry out this technique is more costly than that of standard staining techniques.

Enzyme immunoassay tests (EIA) and enzyme-linked immunosorbent assays (ELISA) can also be used to detect *Cryptosporidium* infection. These techniques are very sensitive in detecting oocyst surface antigens. For ELISA, antibodies are added to the sample and bind to the antigen of the oocyst. A second antibody is

then added, which binds to the complex and this second antibody has an enzyme linked to it which converts the substrate to a coloured product. Colour is, therefore, produced in proportion to the amount of antigen in the sample. These tests are not species specific but have been shown to be more efficient for testing for the presence of oocysts than immunofluorescent tests (Graczyk *et al.*, 1996). However, the cost of these methods is much higher than for microscopy.

Kehl *et al.* (1995) compared 4 methods for the detection of oocysts from human faeces. The authors compared 2 enzyme immunoassays and a direct immunofluorescent assay with acid-fast Kinyoun staining. None of the techniques detected all 55 positive samples present and each method had similar sensitivity and specificity. However, as with fluorescent staining, there tends to be limitations linked to immunological assays, primarily non-specificity due to cross reactivity with other microorganisms (Fayer *et al.*, 2000).

1.5.4 Molecular techniques

A number of molecular tools have been developed as an alternative to (or supplementary to) microscopy and immunological assays, of which the most widely used is polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) of specific gene loci. RFLP detects differences among species by amplifying a sequence, digesting the product with restriction enzymes and analysing banding patterns by gel electrophoresis (Carey *et al.*, 2004). This method is advantageous in that the species of *Cryptosporidium* causing the infection can be determined which can help with tracking the source of infection and identifying risk factors for disease. Under the microscope, most species are morphologically identical except the larger *C. andersoni* and *C. muris* oocysts and so molecular techniques offer great advantages.

Prior to PCR, oocysts must be disrupted in order to release sporozoite DNA. Mechanical disruption in the form of freeze-thawing (FT) and bead beating is often used. Fifteen cycles of FT has been shown to be effective with a sensitivity of >10 oocysts (Johnson *et al.*, 1995; Nichols *et al.*, 2003; Nichols and Smith, 2004; Nichols *et al.*, 2006b). Commonly targeted loci include: SSU rRNA, 70k Da heat shock protein (HSP70) and *Cryptosporidium* oocysts wall protein (COWP). As discussed by Smith (2008), the most robust information on species and genotypes has been obtained from the study of 3 genetic loci: 2 loci on the SSU rRNA gene

(Johnson *et al.*, 1995; Xiao *et al.*, 1999b; Xiao *et al.*, 2001a; Nichols *et al.*, 2003) and the COWP gene (Homan *et al.*, 1999). Although analysis of the COWP gene (Homan *et al.*, 1999) reduces the risk of contamination often generated using nested PCRs, nested assays are essential when oocyst numbers are low. In addition, Jiang and Xiao (2003) indicated that analysis of the SSU rRNA gene loci is superior. They tested the performance of 10 commonly used genotyping tools in the detection and differentiation of 7 *Cryptosporidium* species (C. h*ominis*, *C. parvum*, *C. meleagridis*, *C. felis*, *C. canis*, *C. muris* and *Cryptosporidium* pig genotype 1). Three PCR-RFLP tools targeting the SSU rRNA gene loci amplified and differentiated among all 7 species efficiently. However, the COWP and HSP70 gene loci detected and differentiated *C. hominis*, *C. parvum* and *C. meleagridis* only. Morse *et al.* (2007) compared 3 PCR assays; direct PCR amplification of the SSU rRNA gene locus (Johnson *et al.*, 1995), nested PCR amplification of the SSU rRNA gene locus (Johnson *et al.*, 1995; Nichols *et al.*, 2006a) and single tube nested PCR amplification of the COWP gene locus (Homan *et al.*, 1999). Nested PCR amplification of the SSU rRNA gene was the only method to give 100% positive results. An overall 80% positive result was achieved when the COWP gene was targeted (Morse *et al.*, 2007).

Quantitative real time PCR involves the incorporation of fluorgenic dyes into the amplicon during PCR. As more product is produced, intensity of fluorescence increases, thus producing a quantitative measure of the amount of product present and eliminates the need for post-PCR analysis.

Although PCR-RFLP is rapid, highly sensitive, cost-efficient and can determine species present, there are associated limitations. False positives can result from the detection of naked nucleic acids, non-viable oocysts and laboratory contamination and inhibition cannot be directly assessed (Fayer *et al.*, 2000; Carey *et al.*, 2004).

Various tools for the subtyping of *C. hominis* and *C. parvum* have also been developed. The most common include glycoprotein 60 (GP60) sequencing (Strong *et al.*, 2000; Glaberman *et al.*, 2001; Glaberman *et al.*, 2002; Leav *et al.*, 2002a; Peng *et al.*, 2003a) and minisatellite (MN) and microsatellite (MS) markers (Caccio *et al.*, 2000; Enemark *et al.*, 2002; Mallon *et al.*, 2003). These techniques discriminate subspecies which can aid in disease and source tracking.

1.5.5 Sensitivity of detection

Sensitivity of detection for microscopy is low. For unconcentrated smears, a limit of 10^6/ml has been reported using mZN (Smith, 2008). After stool concentration, fluorescent staining of smears yields detection limits of $>10^4$ oocysts/gram (opg) depending on stool consistency (Weber *et al.*, 1991). Sensitivity improves for IF microscopy but remains low at $>10^3$ opg due to the small amount of material that can be tested and the presence of obscuring debris (Robinson *et al.*, 2008b) and, according to Periera *et al.* (2002), there is a 5% likelihood of generating false negatives.

In a study by Webster *et al.* (1996), oocysts were seeded in bovine faeces and sensitivity of several methods for recovery analysed. Oocysts were not detected in samples seeded with 10,000 oocysts following formol-ether sedimentation and examination using auramine-phenol (AP) or by immunofluorescent (IF) staining. When oocysts were concentrated using sucrose flotation, the threshold of detection was 4000 oocysts per gram for both staining methods. Following salt flotation, 4000 oocysts per gram could be detected reliably by AP staining but the detection limit was increased to 6000 oocysts per gram using IF staining. PCR coupled with IMS detected 5 oocysts per ml of diluted faeces, which corresponds to 80-90 oocysts per gram, indicating that IMS was the most sensitive method available for oocysts detection.

1.6 Transmission

Several factors facilitate the transmission of *Cryptosporidium*: (1) *Cryptosporidium* has wide host specificity, (2) it has a low infectious dose with possibly only 9 oocysts required to cause infection, (3) oocysts are fully sporulated and infectious when shed; and (4) oocysts are robust and can survive for long periods in the environment. Transmission can be direct (host-to-host) or indirect through ingestion of oocysts in contaminated food or water. Thus, transmission routes for *Cryptosporidium* include person-to-person, zoonotic, waterborne and food-borne. The identification of transmission routes and sources of infection has been greatly improved with the development of molecular techniques to identify the species of *Cryptosporidium* causing infections. Species which are host specific will have a

narrow host range and so sources of infection may be determined with greater ease. However, sources of infection and routes of transmission for *C. parvum* can be more difficult to determine owing to the broad host range of this species.

1.6.1 Person-to-person transmission

Direct person-to-person transmission has been documented in nurseries, daycare centres, in homosexual relationships, within hospitals and also within families (Koch *et al.*, 1985; Goodgame *et al.*, 1993; Newman *et al.*, 1994; Fayer *et al.*, 2000; Hellard *et al.*, 2003; Pandak *et al.*, 2006; Turabelidze *et al.*, 2007). However, few studies from tropical regions have investigated direct person-to-person transmission.

In Durban, South Africa, an outbreak of cryptosporidiosis occurred in a day care centre with 73% of children and 10% of staff becoming infected. The outbreak was considered to have occurred predominantly by person-to-person contact between children and their carers (Walters *et al.*, 1988). Likewise, in a paediatric hospital in Mexico, an outbreak of cryptosporidiosis in under-nourished infants was investigated. Although the source of infection was tracked to a single infant, the transmission route could not be determined due to the lack of direct contact between that infant and those who later became infected. It was later established that the hospital staff were most likely the cause of disease spread. When assessed, only 30% of ward staff were washing their hands before attending to an infant (Navarrete *et al.*, 1991).

Although other studies from developing countries have not assessed the possible sources of infection, risk factor analyses have indicated that person-to-person transmission may occur. Crowded living conditions have been highlighted as a risk factor for both children and adults in Venezuala, Indonesia and Brazil (Chacin-Bonilla *et al.*, 1997; Katsumata *et al.*, 1998; Newman *et al.*, 1999). Presumably, crowding would lead to decreased hygiene conditions and increased person-to-person contact, increasing the likelihood of person-to-person transmission. Similarly, the number of children attending daycare and the number of children in the household with diarrhoea (Pereira *et al.*, 2002) were associated with risk of infection in Brazil. This is consistent with risk factor analysis in Australia and the US (Robertson *et al.*, 2002; Hunter *et al.*, 2004), where contact with persons with diarrhoea and toileting a child under 5 years were highlighted as risk factors. In addition, Rahman *et al.* (1990) established person-to-person transmission within

families, with 12.5% of family members studied in Bangladesh contracting cryptosporidiosis 3 weeks following the diagnosis of infection of a child in the household.

1.6.2 Waterborne transmission

Cryptosporidium outbreaks in the developed world have been associated mainly with contaminated drinking water, the most devastating of which occurred in Milwaukee, USA in 1993. Over 400,000 people were infected when untreated water from Lake Michigan which contained oocysts entered the public water supply (Mackenzie *et al.*, 1994). The oocysts were not removed by the coagulation and filtration processes in the water treatment plant and entered the water supply, resulting in the massive outbreak. This incident, in particular, highlighted the need for more stringent control measures in water treatment plants.

Other large waterborne outbreaks have been studied in Texas, Georgia, Oregon, Ontario, British Columbia, Japan and the UK affecting an estimated 2006, 12,960, 1500, >1000, 14,500, 2,097, >9,000 and >4,321 individuals, respectively (Fayer *et al.*, 2000). Oocysts have been identified in both surface and groundwaters. Four to 100% of surface water samples examined from the UK and USA were found to contain oocysts, with concentrations ranging from 0.1-10,000 oocysts/100L (Lisle and Rose, 1995). In addition, 9.5-22% of US groundwater samples tested positive for *Cryptosporidium* (Fayer *et al.*, 2000).

Livestock were believed to be the major source of waterborne cryptosporidiosis and can contaminate soils and pastures with rainfall leading to runoff into streams and rivers. However, with the aid of molecular techniques such as genotyping and subtyping, sources of infection can be identified more accurately and human effluent is now thought to be as, if not more, important than livestock in the contamination of water leading to outbreaks (Hunter and Thompson, 2005). Cryptosporidiosis has also been linked with exposure to recreational water such as swimming pools [owing to diapered children, toddlers and incontinent persons (Joce *et al.*, 1991; Ichinohe *et al.*, 2005; Causer *et al.*, 2006)], freshwater lakes (Yoder *et al.*, 2004) and fountains (CDC, 1998; Eisenstein *et al.*, 2008). From January 2005 to December 2006, 40% of waterborne disease outbreaks in the USA associated with recreational water were caused by *Cryptosporidium*. 83% of these occurred in swimming pools and 17% in freshwater (Yoder *et al.*, 2008).

In developing countries, rivers and lakes are often used for activities such as bathing, washing clothes and obtaining drinking water and so it is likely that there is an increased risk of infection from these sources in such countries. However, there is a general lack of data from developing regions relating to waterborne outbreaks, and this may be attributed to a lack of surveillance in developing countries coupled with the endemic nature of the infection and the low level of immunity against infection in people in these regions (Newman *et al.*, 1994; Zu *et al.*, 1994).

1.6.3 Foodborne transmission

Cryptosporidium may also be transmitted via consumption of contaminated food. According to Smith *et al.* (2006), there is an increased risk of oocyst contamination with the rise of global sourcing and rapid transport of food. Approximately 10% of all cryptosporidiosis cases in USA are thought to be foodborne, accounting for 64% of the estimated 357,000 US cases of parasitic foodborne illness (~700 hospitalisations and 8 deaths) (Smith and Grimason, 2005).

Contamination of food can occur during a number of stages of food production. Crops can be contaminated when grown in soils containing oocysts and/or if irrigated with contaminated water. In addition, contamination may occur owing to unhygienic handling of food during transportation and food preparation and/or contact with contaminated surfaces. Cooking food is likely to render the oocysts non-infectious but foods that are not further treated such as salads, fruits and raw milk are high-risk sources of infection.

Several food-associated outbreaks have been documented including contamination of cider (Millard *et al.*, 1994; Blackburn *et al.*, 2006), pasteurised milk (Gelletlie *et al.*, 1997; Harper *et al.*, 2002), salad buffet (Ethelberg *et al.*, 2009) and chicken salad (CDC, 1996). In a suburban slum of Lima, Peru, basil, cabbage, celery, cilantro, green onions, ground green chilli, leeks, lettuce, parsley and yerba buena from several market places were contaminated with oocysts of *C. parvum* (Ortega *et al.*, 2006). It has been well documented that freshwater and marine shellfish (e.g. mussels, clams and oysters) concentrate oocysts in contaminated water and because oocysts can survive for up to a year in sea water and 14 days in molluscs (Tamburrini and Pozio, 1999) these are also potential vehicles of transmission (Fayer *et al.*, 2004). However, neither infections nor outbreaks have been

reported from this medium. As in the case of waterborne transmission in developing countries, food-related infections and outbreaks are underreported and this is likely to be due to poor laboratory methods and surveillance.

1.6.4 Zoonotic transmission

Zoonotic transmission of infective oocysts from animal to human hosts may be by direct or indirect transfer. Direct transmission was documented when young children and farm workers came into contact with infected cattle (Rahman *et al.*, 1985; Dawson *et al.*, 1995; Smith *et al.*, 2004; Gait *et al.*, 2008; Hoek *et al.*, 2008). This route of transmission was also highlighted in the UK when, in 2001, strict restrictions on countryside access and movement of livestock was imposed due to the outbreak of Foot and Mouth disease. These restrictions reduced the number of humans coming in contact with livestock and a dramatic decrease (up to 63%) in the incidence of *C. parvum* occurred as a result (Hunter *et al.*, 2003; Smerdon *et al.*, 2003). In addition Hunter *et al.* (2004) highlighted touching farm animals as a risk factor associated with *C. parvum* infection.

Cattle have been repeatedly implicated as the major source of contaminated waters, however genotyping data has often revealed that human effluent is the source of infection (Hunter and Thompson, 2005). According to Hunter and Thompson (2005), cattle were not identified as a source of any waterborne outbreak in the US, and in Canada an outbreak in Cranbrook was the only 1 associated with *C. parvum* oocysts. Because *C. hominis* is host specific and only identified on a limited number of occasions infecting species other than humans [cattle in Scotland, India and Korea (Smith *et al.*, 2005b; Park *et al.*, 2006), a goat and a sheep in the UK (Giles *et al.*, 2009) and a dugong (Morgan *et al.*, 2000c)], human effluent was most likely the cause of the other outbreaks (Hunter and Thompson, 2005). In a study undertaken in England, it was found that *C. parvum* was the causative agent in 84% (56/67) of *Cryptosporidium* cases, and the authors indicated that livestock faecal pollution of water sources was the leading cause of human sporadic cryptosporidiosis (Goh *et al.*, 2004). However, with the recent development of subtyping tools, it has been shown that not all subtypes of *C. parvum* infect both humans and cattle. Variants which are predominantly or exclusively associated with humans but not animals have been identified (Alves *et al.*, 2003).

Therefore, both species and subspecies data is required before conclusions regarding the source of infection can be made. Similarly, pets were once believed to be important sources of infection for humans. However, species infecting dogs and cats are generally the host adapted species, *C. canis* and *C. felis*, respectively (Abe *et al.*, 2002) and although these have been identified in human infections (Guyot *et al.*, 2001; Xiao *et al.*, 2001a; Gatei *et al.*, 2002b; Cama *et al.*, 2003; Gatei *et al.*, 2006a; Ajjampur *et al.*, 2007; Cama *et al.*, 2007; Gatei *et al.*, 2007) transmission directly from a pet to a human has not been proven conclusively. Although possible associations between human cryptosporidiosis and contact with dogs have been identified (Xiao *et al.*, 2007b) conflicting studies have found no association (Goh *et al.*, 2004) and have even found contact to be protective (Robertson *et al.*, 2002). This highlights the need for genetic characterisation when aiming to determine sources of infection. A pet's potential to act as a zoonotic reservoir or mechanical vector cannot be overlooked but this mode of transmission is likely to be of lower importance.

1.6.5 Mechanical transmission

Insects such as filth flies and dungbeetles may act as mechanical vectors for the spread of *Cryptosporidium* oocysts. In an experimental setting, it was shown that the housefly can transmit oocysts from bovine faeces to food (Graczyk *et al.*, 1999b; Graczyk *et al.*, 1999a). Oocysts have also been found on flies in a cattle barn (Graczyk *et al.*, 2000) and on dungbeetles (Mathison and Ditrich, 1999).

1.6.6 Airborne transmission

According to Fayer *et al.* (2000) no proven cases of airborne transmission in humans has been documented but there are numerous reports of high rates of cough or other pulmonary symptoms in children and immunocompromised people with *Cryptosporidium*. Although lethal respiratory cryptosporidiosis has been reported for persons with AIDS, the occurrence of respiratory cryptosporidiosis is rarely reported (Tzipori and Ward, 2002).

1.7 Epidemiology in tropical environments

1.7.1 Prevalence and seasonality

Cryptosporidium is a ubiquitous parasite. It has been described in over 40 countries ranging from tropical to temperate zones and including both the developed and the developing world (Kosek *et al.*, 2001). Numerous studies have been carried out evaluating the prevalence of *Cryptosporidium* in the stool samples of various populations. These studies vary widely in terms of geographical location, hospital or community-based studies, age of participants, sample size, immune status of participants (HIV infection, malnourishment) and clinical status (presence of diarrhoea) and so, it is difficult to compare prevalence data from a range of studies.

Fayer (2004) states that the most thorough review of geographical distribution and prevalence of *Cryptosporidium* infection in humans was compiled by Ungar (1990). More than 100 surveys from 40 countries were reviewed. Based on oocysts in faecal specimens, the prevalence of human infection in African countries was 2.6 – 21.3%, in Central and South American countries, 3.2 – 31.5% and Asian countries, 1.3 – 13.1%. In contrast, the prevalence in Europe and North America were found to be 0.1 – 14.1% and 0.3 - 4.3% respectively. Although Ungar's study was carried out almost 2 decades ago, according to Fayer (2004), more recent surveys from other locations indicate similar rates of prevalence. O'Donoghue (1995) also grouped prevalence data for both the developing and the developed world (excluding reports on AIDS patients and specific outbreaks). Prevalence ranged from 0.1 – 27.1% in developed, industrialised areas (mean of 56 surveys = 4.9%) compared with 0.1-31.5% in the less developed countries (mean of 48 surveys = 7.9%). The prevalence of infection in asymptomatic individuals ranges from 0 – 2% in the developed world (mean of 12 surveys = 0.3%) compared with 0 – 9.8% in the developing world (mean 20 of surveys = 1.6%). From these data it is clear that the prevalence rates are much greater in developing countries in comparison with the developed world. Better sanitation, cleaner drinking water, less overcrowding and possibly less contact with domestic animals in more developed countries are most likely to account for the differences (Ungar, 1990; O'Donoghue, 1995; Fayer, 2004).

Table 1.5 presents the prevalence of *Cryptosporidium* infection from a number of tropical countries and highlights the differences in design between these various studies. From this table, prevalence of *Cryptosporidium* infections in tropical countries (adults and children combined) with diarrhoea ranges from 0-39% (mean of 34 studies: 11.8%) and those without diarrhoea ranges from 0-31.6% (mean of 22 studies: 4.5%) (asymptomatic) (excluding the study from Gay-Andrieu *et al.* (2007) which had only 7 non-diarrhoeic cases). Reported prevalence of childhood infection in developing countries such as Kenya, Uganda, Nigeria, Guatemala, Jordan, Pakistan, Egypt, Brazil, Indonesia, Malawi, Peru, Chile, Bolivia, Bangladesh, India, Niger, Mexico and Gambia ranges from 0% – 32% (Table 1.5). Although the majority of reports incorporate information about diarrhoeal status, many do not test for HIV infection and malnutrition, and very few assess the prevalence of *Cryptosporidium* infection in HIV/AIDS patients without diarrhoea.

To date, 10 studies have been conducted in Nigeria focusing on the prevalence of *Cryptosporidium* infection in various populations (Reinthaler *et al.*, 1987; Kwaga *et al.*, 1988; Oyerinde *et al.*, 1989; Okafor and Okunji, 1994; Okafor and Okunji, 1996; Nwabuisi, 2001; Nwokediuko *et al.*, 2002; Banwat *et al.*, 2003; Okodua *et al.*, 2003). From these 10 studies (Table 1.5), prevalence ranged from 0%-39%. Of the 8 studies containing information on diarrhoeal status, the mean prevalence of *Cryptosporidium* in individuals with diarrhoea is 12.8% (8 studies: Range: 0-39%) while those without diarrhoea was 4.8% (6 studies: Range: 0-24%). However, no study from Nigeria has characterised the species of *Cryptosporidium* present nor have they explored risk factors for infection.

In addition to variations in prevalence rates among countries, prevalence also varies temporally, with higher rates reported during the rainy season in many tropical areas (Adegbola *et al.*, 1994; Javier Enriquez *et al.*, 1997; Newman *et al.*, 1999) (Table 1.5). An increase in rainfall presumably leads to the greater spread of contaminated water (Dillingham *et al.*, 2002). A longitudinal study of *Cryptosporidium* infection in children was conducted in Northeast Brazil and it was found that *Cryptosporidium* was highly seasonal, with a peak in months with the highest rainfall (Newman *et al.*, 1999). This was consistent with findings from Mexico, Brazil, Kuwait, Indonesia, India, Gambia, Uganda and Malawi (Adegbola *et al.*, 1994; Javier Enriquez *et al.*, 1997; Katsumata *et al.*, 1998; Iqbal *et al.*, 1999; Pereira *et al.*, 2002; Peng *et al.*, 2003b; Tumwine *et al.*, 2003; Das *et al.*, 2006; Morse *et al.*, 2007). However, other studies have provided conflicting results. In Kenya, prevalence was highest during the dry season that follows the short rains (Gatei *et al.*, 2006a), while in Venezuela, Guatemala and Egypt no seasonal

variation for infection was detected (Chacin-Bonilla *et al.*, 1997; Laubach *et al.*, 2004; Abdel-Messih *et al.*, 2005) (Table 1.5).

Table 1.5 Prevalence and seasonality of *Cryptosporidium* infection in developing countries

Location	Age Group	Sample Size	Detection technique	Diarrhoeal status	HIV status	Prevalence	Seasonality	Reference
Osun State, SW Nigeria (Community)	1-6 yrs	1636 children 3480 samples	Formal-ether (FE) concentration & auramine phenol (AP) staining	Unknown	Unknown	From children's 1st samples: 15.2%	Peak in May (not associated with rainfall)	See current thesis
Ogun state, SW Nigeria (Hospital & Community)	Children & adults	479	Teleman concentration & Modified Ziehl Neelson (mZN) stain	420 diarrhoeic 59 non-diarrhoeic	Unknown	Diarrhoeic: 2.3% Non-diarrhoeic: 0%	Unknown	Reinthaler *et al.* (1987)
Enugu state, SE Nigeria (Hospital)	15-56 yrs	189	Formal-ether (FE) concentration & mZN	All diarrhoeic	HIV+: 85.2% HIV-: 14.8%	HIV+: 0% HIV-: 0%	Unknown	Nwokediuko *et al.* (2002)
Enugu state, SE Nigeria (Hospital)	Primary school children	373	Formalin ethyl acetate concentration & Safranin-methelene blue stain	335 formed stools 38 watery stools	Unknown	Formed: 24% Watery: 39%	Unknown	Okafar and Okunji (1996)
Kwara state, W Nigeria (Community)	Children (0-14yrs)	301	FE concentration & Safranin-methelene blue stain	198 diarrhoeic 103 non-diarrhoeic	Unknown	Diarrhoeic: 15.1% Non-diarrhoeic: 0%	Unknown	Nwabuisi (2001)

Table 1.5 Contd

Location	Age Group	Sample Size	Detection technique	Diarrhoeal status	HIV status	Prevalence	Seasonality	Reference
Abeokuta, SW Nigeria (Hospital)	Children & adults	215	FE concentration & mZN stain	Unknown	HIV+: 35 HIV-: 180	HIV+: 11.1% HIV-: 0%	Unknown	Okodua *et al.* (2003)
Lagos, SW Nigeria (Hospital)	Children & adults	890	Formol saline ether concentration, mZN, Safranin-methelene blue stain	Diarrhoeic & non-diarrhoeic	Unknown	Overall: 0%	No infection	Oyerinde *et al.* (1989)
Kaduna state, N Nigeria (Hospital)	Children & adults	75	FE concentration & Safranin-methelene blue stain	All diarrhoeic	Unknown	Overall: 21% Adults: 29% Children: 8%`	Unknown	Kwaga *et al.* (1988)
Jos, Central Nigeria (Hospital)	0-3.5 yrs (Undernourished children)	104	mZN stain	Unknown	HIV +: 50% HIV -: 50%	HIV+: 0% HIV-: 3.8%	Unknown	Banwat *et al.* (2003)
Former Anambra state, S Nigeria (Hospital)	Children & adults	433	Formalin ethyl acetate concentration & Safranin-methelene blue	413 diarrhoeic 30 non-diarrhoeic	Unknown	Diarrhoeic: 12.5% Non-diarrhoeic: 0.03%	Unknown	Okafar and Okunji (1994)
Niamey, Niger (Hospital)	Children (<5yrs)	220	Direct stool examination, Ritchie technique & mZN	113 diarrhoeic 7 non-diarrhoeic	Unknown	Overall: 5.5% Diarrhoeic: 6.2% Non-diarrhoeic: 71.4%	Unknown	Gay-Andrieu *et al.* (2007)

Table 1.5 Contd

Location	Age Group	Sample Size	Detection technique	Diarrhoeal status	HIV status	Prevalence	Seasonality	Reference
Gambia (Hospital & community)	Children (<5yrs)	Hospital: 1200 (600 cases, 600 controls) Community: 250	FE Concentration & mNZ	Hospital: 600 diarrhoeic 600 non-diarrhoeic Community: 250	Unknown	Hospital: Diarrhoeic: 9% Non-diarrhoeic: 3% Community: 4%	Peak in rainy season (July-Aug)	Adegbola *et al.* (1994)
Kenya (Community)	Children (<5yrs)	4899	mZN stain PCR-RFLP	All diarrhoeic	Unknown	Overall: 4%	Peak in dry season following rainy (Nov-Feb)	Gatei *et al.* (2006a)
Egypt (Hospital)	Children (<5yrs)	1275	ELISA	All diarrhoeic	Unknown	Overall: 17%	No seasonal variation	Abdel-Messih *et al.* (2005)
Uganda (Hospital)	Children (<5 yrs)	243	PCR-RFLP	All diarrhoeic	HIV+: 91 HIV-: 152	Overall: 31.3% HIV+: 73.6% HIV-: 5.9%	Unknown	Tumwine *et al.* (2005)
Uganda (Hospital)	Children (<5yrs)	2446	Acid-fast staining PCR-RFLP	1779 diarrhoeic 667 non-diarrhoeic	Unknown	Diarrhoeic: 25% Non-diarrhoeic: 8.5%	Peak in rainy season (April-June)	Tumwine *et al.* (2003)
Tanzania (Hospital)	Adults	127	IF microscopy PCR-RFLP	61 diarrhoeic 66 non-diarrhoeic	All HIV+	Diarrhoeic:18% Non-diarrhoeic: 16.7%	Unknown	Houpt *et al.* (2005)

Table 1.5 Contd

Location	Age Group	Sample Size	Detection technique	Diarrhoeal status	HIV status	Prevalence	Seasonality	Reference
Zambia (Community)	Adults	289	ELISA PCR	All diarrhoeic	Unknown	Overall: 6%	Unknown	Siwila *et al.* (2007)
Malawi (Hospital)	Children (1-30mo)	96	Acid-fast stain PCR-RFLP	Unknown	HIV+: 13 HIV-: 45 Unknown: 11	Unknown	Peak in rainy season	Peng *et al.* (2003b)
S. Africa (Hospital & Community)	Hospital: Children & adults (1mo-88yrs) Community: Children (3-15yrs)	244	Real time PCR PCR-RFLP	197 diarrhoeic 47 non-diarrhoeic	HIV+: 31 (All with diarrhoea)	Overall: 18%(44/244) Diarrhoeic: 9.8% Non-diarrhoeic: 8.2%	Unknown	Samie *et al.* (2006)
Calcutta, India (Hospital)	Children & adults	563 (289 cases 274 controls)	Kinyouns acid-fast stain	289 diarrhoeic 274 non-diarrhoeic	Unknown	Diarrhoeic: 5.5% Non-diarrhoeic: 1.1%	Unknown	Das *et al.* (1993)
Bangladesh (Hospital)	Children (<5yrs)	1672	Modified acid-fast stain	All diarrhoeic	Unknown	Overall: 2.6%	Unknown	Khan *et al.* (2004)

Table 1.5 Contd

Location	Age Group	Sample Size	Detection technique	Diarrhoeal status	HIV status	Prevalence	Seasonality	Reference
S. India (Community	Children (0-5yrs)	1,949	Acid-fast stain	All diarrhoeic	Unknown	Overall: 7.6%	Unknown	Ajjampur *et al.* (2007)
India (Hospital)	Children (<5yrs)	257	Modified acid-fast stain	158 diarrhoeic 99 non-diarrhoeic	Unknown	Diarrhoeic: 13.3% Non-diarrhoeic: 2.04%	Unknown	Das *et al.* (2006)
Kolkata, India (Comminuty)	Children (0-12yrs)	609	Kinyouns acid-fast stain	None diarrhoeic	Unknown	Overall: 2.3%	Unknown	Palit *et al.* (2005)
Pakistan (Hospital)	Children (<5yrs)	625	Formalin ethyl acetate concentration & methelene blue stain	475 diarrhoeic 150 non-diarrhoeic	Unknown	Diarrhoeic: 10.3% Non-diarrhoeic: 3.3%	Unknown	Iqbal *et al.* (1999)
Kuwait (Community)	Children (3mo-13yrs)	1009 (509 cases 500 controls)	FE concentration & modified Safranin-methelene blue stain	509 diarrhoeic 500 non-diarrhoeic	Unknown	Diarrhoeic: 10% Non-diarrhoeic: 0%	Peak in Jan and April. No rainy season peak	Iqbal *et al.* (2001)

Table 1.5 Contd

Location	Age Group	Sample Size	Detection technique	Diarrhoeal status	HIV status	Prevalence	Seasonality	Reference
Indonesia (Hospital & community)	Children (<10yrs)	Hospital: 917 cases 1043 controls Community: 4368	Sugar flotation & modified Kinyouns acid-fast stain	Hospital: 917 diarrhoeic 1043 non-diarrhoeic	Unknown	Hospital: Overall: 2.1% Diarrhoeic: 2.8% Non-diarrhoeic: 1.4% Community: Overall: 1.1% Diarrhoeic: 8.2% Non-diarrhoeic: 0.7%	*Hospital*: Peak in rainy season *Community*: Unknown	Katsumata *et al.* (1998)
Jordan (Hospitals)	Children (6mo-6yrs)	600 (300 cases 300 controls)	Sheathers flotation & acid-fast staining	300 diarrhoeic 300 non-diarrhoeic	Unknown	Diarrhoeic: 6.7% Non-diarrhoeic: 1.7%	Unknown	Nimri *et al.* (1994)
Mexico (Hospital)	Children (<5yrs)	403	Sugar flotation, acid-fast kinyoun stain & Indirect immunoflourescence	All diarrhoeic	100 tested & all HIV-	Overall: 6.4%	Peak in rainy season	Javier-Enriquez *et al.* (1997)
Mexico (Hospital & community)	Children (<5yrs)	166	Saline concentration & anti-oocyst wall monoclonal antibody based immunofluorescence	All diarrhoeic	Unknown	Overall: 19.3% Hospital (Urban): 29.6% Community (Rural): 9.4%	Unknown	Miller *et al.* (1994)

Table 1.5 Contd

Location	Age Group	Sample Size	Detection technique	Diarrhoeal status	HIV status	Prevalence	Seasonality	Reference
Guatemala (Community)	Children (2-13yrs)	100	Kinyoun modified acid-fast stain	All diarrhoeic	Unknown	Overall: 32%	No seasonal variation	Laubach *et al.* (2004)
Zulia state, Venezuala (Hospital)	Children (<5yrs)	460 (310 cases, 150 controls)	mZN carbolfushin stain	310 diarrhoeic 150 non-diarrhoeic	Unknown	Diarrhoeic: 11.2% Non-diarrhoeic: 6%	No seasonal variation	Chacin-Bonilla *et al.* (1997)
Lima, Peru (Hospital)	Adults (18-67yrs)	2672	Acid-fast stain PCR-RFLP	Unknown	All HIV+	Overall: 13.3%	Unknown	Cama *et al.* (2003)
Peru (Community)	Children (1mo-9yrs)	489	QIAamp DNA mini kit PCD-RFLP	147 Diarrhoeic 342 non-diarrhoeic	Unknown	Diarrhoeic: 3.4% Non-diarrhoeic: 0%	Unknown	Cordova Paz Soldon *et al.* (2006)
Peru (Hospital)	Adults (>17 yrs)	2490	Modified Ritchie formalin ether concentration & modified acid-fast stain	Diarrhoeic & non-diarrhoeic	All HIV+	Overall: 9.2% (230/2490)	Unknown	Cama *et al.* (2007)
Brazil (Hospitals)	Children (2wks-10yrs)	445	Direct immunofluorescent assay	All diarrhoeic	HIV+: 0	Overall: 18.7%	Peak in late rainy season	Pereira *et al.* (2002)

Table 1.5 Contd

Location	Age Group	Sample Size	Detection technique	Diarrhoeal status	HIV status	Prevalence	Seasonality	Reference
Brazil (Community)	Children (<4yrs)	189	FE concentration & AP stain	All diarrhoeic	Unknown	Overall: 31.2%	Peak in rainy season	Newman *et al.* (1999)
Bolivia (Community)	Children (5-19yrs)	377	FE concentration & mZN stain	None diarrhoeic	Unknown	Overall: 31.6%	Unknown	Esteban *et al.* (1998)
Chile (Hospital)	Children & adults	68	ZN stain PCR-RFLP	All diarrhoeic	HIV+: 27 Malnourished children: 27 Immunocompetant adults & children: 14	Overall: 5.9% HIV+ Adults: 11.1% Malnourished children: 0% Immunocompetant: 14.3%	Unknown	Neira-Otera *et al.* (2005)

1.7.2 Species, genotypes and subtypes

Understanding which species/genotypes of *Cryptosporidium* are present in various geographical areas is essential as isolates from different regions may have different antigens, virulence and infectivity (Fayer and Ungar, 1986; Fayer *et al.*, 2000; Cordova Paz Soldon *et al.*, 2006; Bushen *et al.*, 2007; Cama *et al.*, 2007). Species which have been identified in tropical regions to date include *C. hominis*, *C. parvum*, *C. felis*, C. canis, *C. muris*, *C. meleagridis* and *C. suis* (A putative case of *C. andersoni* was also detected in Malawi (Morse *et al.*, 2007)) (Table 1.6). In Africa, genotyping data has been obtained from a limited number of countries, namely Uganda, Malawi, Kenya, Egypt and S. Africa and species identified include all species mentioned above with the exception of *C. suis* (although this is not to say that this species does not exist in African countries, just that no study to date has identified it).

C. parvum and *C. hominis* are the most common species infecting humans. *C. parvum* can infect a range of hosts including humans whereas *C. hominis* primarily infects human hosts [although there are sporadic reports of *C. hominis* infections in other animals (Morgan *et al.*, 2000c; Smith *et al.*, 2005b; Giles *et al.*, 2009)]. Studies from Peru (Xiao *et al.*, 2001a; Cordova Paz Soldon *et al.*, 2006), Kenya (Gatei *et al.*, 2003; Gatei *et al.*, 2006a), India (Das *et al.*, 2006; Ajjampur *et al.*, 2007; Gatei *et al.*, 2007), Malawi (Peng *et al.*, 2003b; Morse *et al.*, 2007) and Uganda (Tumwine *et al.*, 2003; Akiyoshi *et al.*, 2006) have shown the ubiquitous presence of *C. hominis* and *C. parvum* infection and indicate that *C. hominis* is the dominant species present in these areas. This implies that anthroponotic transmission may play a major role in the epidemiology of *Cryptosporidium* in these regions. In contrast, higher levels of *C. parvum* are consistent with studies from Kuwait, Equatorial Guinea and developed countries such as the UK, Portugal and France (McLaughlin *et al.*, 2000; Guyot *et al.*, 2001; Alves *et al.*, 2003; Sulaiman *et al.*, 2005; Blanco *et al.*, 2009).

Subtyping data from tropical countries has been obtained from just 9 studies which were conducted in Malawi, Uganda, S. Africa, India, Kuwait and Peru (Table 1.6). The subtypes of *C. hominis* which have been identified are Ia, Ib, Id and Ie and of *C. parvum* IIa, IIc, IId, IIe, IIf, IIg, IIh, and IIi (Table 1.6). As discussed in Section 1.4.2.11, recent subtyping data has indicated that not all *C. parvum* infections in humans are a result of zoonotic transmission. IIa and IIc are responsible for most *C. parvum* infections in humans and distribution varies by geographic regions (Xiao and Ryan, 2008). IIa has been identified in both humans

and ruminants while IIc has been detected only in humans (McLaughlin *et al.*, 2000; Guyot *et al.*, 2001; Alves *et al.*, 2003; Peng *et al.*, 2003b; Xiao and Ryan, 2004; Sulaiman *et al.*, 2005; Alves *et al.*, 2006; Areeshi *et al.*, 2008), indicating that the IIc subtype of *C. parvum* may be exclusive to humans and involved in anthroponotic transmission.

IIe, another anthroponotic *C. parvum* subtype, has been isolated in Malawi and S. Africa (Leav *et al.*, 2002a; Peng *et al.*, 2003b) while IId has been identified in 6 AIDS patients, 3 cows in Portugal (Alves *et al.*, 2003) and children in Kuwait (Sulaiman *et al.*, 2005) . Several new *C. parvum* subtypes have been found to infect humans. IIg, IIh and IIi were isolated from children in Uganda (Akiyoshi *et al.*, 2006), IIl from adults in Slovenia (Soba and Logar, 2008) and IIm from Bangledeshi children (Hira *et al.*, Unpublished, Accession no. AY700401).

The differences in the distribution of *Cryptosporidium* species and subtypes in humans are considered an indication of differences in sources of infection (Sulaiman *et al.*, 2005) and so additional data are required to determine the host ranges of the various species and subtypes.

Table 1.6 *Cryptosporidium* species, genotypes and subtypes isolated in developing countries

Location	Species	Subtypes	Age Group	% positive (number)	HIV status	Reference
Nigeria	*C. hominis*	Ia (10/28) Ib (10/28) Id (4/28) Ie (3/28) Ih (1/28)	11-80 months	43.6 (34/78)	Unknown	See current thesis
	C. parvum	IIa (2/23) IIc (17/23) Iii (2/23) IIm (2/23)		32.1 (25/78)		
	Mixed- *C. hominis/C. parvum*			4/78 (5.1)		
	C. meleagridis	Type 1 (3/3)		6.4 (5/78)		
	Cryptosporidium rabbit genotype			6.4 (5/78)		
	Cryptosporidium cervine genotype			3.9 (3/78)		
	C. canis			1.3 (1/78)		
	Unknown			1.3 (1/78)		
Kenya	*C. hominis*	No subtyping data	Unknown	66.6 (4/6)	All HIV+	Morgan *et al.* (2000b)
	C. parvum			16.7 (1/6)		
	C. meleagridis			16.7 (1/6)		
Kenya	*C. hominis*	No subtyping	Children & adults	39.4 (23/34)	HIV+ & HIV-	Gatei *et al.* (2003)
	C. parvum			23.5 (8/34)		
	C. meleagridis			2.9 (1/34)		
	C. muris			2.9 (1/34)		
Uganda	*C. hominis*	No subtyping	Children (<5 yrs)	73.7 (56/76)	HIV+: 67 HIV-: 9	Tumwine *et al.* (2005)
	C. parvum			18.4 (14/76)		
	C. hominis/C. parvum			3 .9 (3/76)		
	C. meleagridis			3.9 (3/76)		

Table 1.6 Contd

Location	Species	Subtypes	Age Group	% positive (number)	HIV status	Reference
Uganda	*C. hominis*	No subtyping	Children (<5 yrs)	73.7 (326/444)	Unknown	Tumwine *et al.* (2003)
	C. parvum			19.2 (85/444)		
	Mixed- *C. hominis/C. parvum*			4.2 (19/444)		
	C. meleagridis			1.1 (5/444)		
	Unknown			3 (14/444)		
Uganda	*C. hominis*	Ia (4/13) Ib (1/13) Id (1/13) Ie (7/13)	Children (2-84 mo)	74.4 (61/82)	HIV+ & HIV-	Akiyoshi *et al.* (2006)
	C. parvum	IIc (10/15) IIg (1/15) IIh (1/15) IIi (3/15)		18.3 (15/82)		
	C. hominis/C. parvum			3.7 (3/82)		
	C. meleagridis			3.7 (3/82)		
Equatorial Guinea	*C. parvum*	No subtyping	Children & adults	52.9 (18/34)	HIV+ & HIV-	Blanco *et al.* (2009)
	C. hominis			44.1 (15/34)		
	C. meleagridis			2.9 (1/34)		
Malawi	*C. hominis*	No subtyping	Children (<5 yrs)	58.1 (25/43)	Unknown	Morse *et al.* (2007)
	C. parvum			23.3 (10/43)		
	C. hominis/C. parvum			2.3 (1/43)		
	C. hominis OR C. parvum			9.3 (4/43)		
	C. meleagridis			4.7 (2/43)		
	C. andersoni			2.3 (1/43)		

Table 1.6 Contd

Location	Species	Subtypes	Age Group	% positive (number)	HIV status	Reference
Malawi	*C. hominis*	Ia (4/26) (1 subtype) Ib (5/26) (1 subtype) Id (11/26) (3 subtypes) Ie (6/26) (1 subtype)	Children (1-30mo)	95.4 (41/43)	Unknown	Peng *et al.* (2003b)
	C. parvum	IIc (1/2) (1 subtype) IIe (1/2) (1 subtype)		4.6 (2/43)		
	C. felis			5.2 (3)		
	C. muris			1.7 (1)		
Malawi	*C. hominis*	No subtyping	Children	63.6 (7/11)	HIV+ & HIV-	Gatei *et al.* (2003)
	C. parvum			36.4 (4/11)		
S. Africa	*C. hominis*	Ia (1/20, 5%) Ib (4/20, 20%) Id (5/20, 25%) Ie (5/20, 25%)	Children (6-36mo)	100 (22/22)	All HIV+	Leav *et al.* (2002a)
	C. parvum	IIc (5/20, 25%)				
S. Africa	*C. hominis*	No subtyping	Children & adults (0-88yrs)	81.8 (36/44)	3 HIV+	Samie *et al.* (2006)
	C. parvum			18.2 (8/44)	1 HIV+	
Kolkatta, India	*C. hominis*	Ia (16.2%) (4 subtypes) Ib (34.9%) (1 subtype) Id (37.2%) (2 subtypes) Ie (12.5%) (1 subtype)	Children	94 (47/50)	Unknown	Gatei *et al.* (2007)
	C. felis			2 (1/50)		
	C. hominis/C. meleagridis			4 (2/50)		

Table 1.6 Contd

Location	Species	Subtypes	Age Group	% positive (number)	HIV status	Reference
Kolkatta, India	*C. hominis*	No subtyping	Children (<5 yrs)	87.5 (35/40)	Unknown	Das *et al.* (2006)
	C. parvum			10 (4/40)		
	C. felis			2.5 (1/40)		
South India	*C. hominis*	Ia (74.5%) Ib (1.7%) Id (17%) Ie (6.4%)	Children (0-3 yrs)	81 (47/58)	Unknown	Ajjampur *et al.* (2007)
	C. parvum	IIc (100%)		12.1 (7/58)		
	C. felis			5.2 (3/58)		
Vietnam	*C. hominis*	No subtyping	Adults	100 (3/3)	HIV+	Gatei *et al.* (2003)
South India	*C. hominis*	Ia (19.3%) Ib (25.8%) Ic (9.7%) Id (12.9%) If (25.8%) Ig (11.1%) Ic + Id (3.2%)	Adults	64.6 (31/48)		Muthusamy *et al.* (2006)
	C. parvum	IIa or IIb (44.4%) IIc (44.4%)		18.8 (9/48)		
	C. felis			10.4 (5/48)	HIV +	
	C. parvum (mouse/ferret)			2.1 (1/48)		
	C. meleagridis			2.1 (1/48)		
	C. muris			2.1 (1/48)		

Table 1.6 Contd

Location	Species	Subtypes	Age Group	% positive (number)	HIV status	Reference
Kuwait	*C. parvum*	IIa (27/58) (2 subtypes) IId (29/58) (5 subtypes) IIc (2/58) (1 subtype) IIf (1/58)	Children (1-19 yrs)	93.6 (58/62)	Unknown	Sulaiman *et al.* (2005)
	C. hominis	Ib (1/3) (2 subtypes) Id (1/3) Ie (1/3)		4.8 (3/62)		
	C. parvum/C. hominis			1.6 (1/62)		
Chile	*C. hominis*	No subtyping	Children & adults	50 (2/4)	HIV+: 27 Malnourished children: 27 Immunocompetant adults & children: 14	Neira-Otera *et al.* (2005)
	C. parvum			50 (2/4)	Unknown	
Peru	*C. hominis*	No subtyping	Children	78.3 (65/83)	HIV-	Xiao *et al.* (2001a)
	C. parvum			9.6 (8/83)		
	C. canis			2.4 (2/83)		
	C. meleagridis			8.4 (7/83)		
	C. felis			1.2 (1/83)		
Peru	*C. hominis*	No subtyping	Adults (18-67 yrs)	67.5 (204/299)	All HIV+	Cama *et al.* (2003)
	C. parvum			11.3 (34/299)		
	C. meleagridis			12.6 (38/299)		
	C. canis			4 (12/299)		
	C. felis			3.3 (10/299)		
	C. suis			0.5 (1/299)		

Table 1.6 Contd

Location	Species	Subtypes	Age Group	% positive (number)	HIV status	Reference
Peru	*C. hominis*	Ia (35/141) (9 subtypes) Ib (39/141) (2 subtypes) Id (40/141) (4 subtypes) Ie (13/141) (1 subtypes)	Adults (>17yrs)	71.2 (141/193)	All HIV+	Cama *et al.* (2007)
	C. parvum	IIc (22/22) (3 subtypes)		11.1 (22/193)		
	C. meleagridis			8.6 (17/193)		
	C. canis			3 (6/193)		
	C. felis			3 (6/193)		
	C. suis			0.5 (1/193)		
Peru	*C. hominis*	No subtyping	Children (1mo-9yrs)	80 (4/5)	Unknown	Cordova Paz Soldon *et al.* (2006)
	C. parvum			20 (1/5)		
Peru	*C. hominis*	Ia (21/78) (6 subtypes) Ib (23/78) (1 subtype) Id (12/78) (3 subtypes) Ie (19/78) (1 subtype) Ib + Ie (1/78) Ib + Id (1/78) Id +Ie (1/78)	Children (0-4yrs)	70.1 (89/127)	Unknown	Cama *et al.* (2008)
	C. parvum	IIc (14/14) (3 subtypes)		13.4 (17/127)		
	C. meleagridis			7.9 (10/127)		
	C. canis			1.6 (2/127)		
	C. felis			4.7 (6/127)		
	C. hominis/C. parvum			1.6 (2/127)		
	C. canis/C. meleagridis			0.8 (1/127)		

1.7.3 HIV/AIDS

Individuals infected with HIV/AIDS are a high-risk group for *Cryptosporidium* infection and have been studied in various geographical locations. From the 7 studies which incorporated information on HIV status in Table 1.5, prevalence of infection in HIV-positive individuals ranged from 0% in Nigeria to 73.6% in Uganda (Nwokediuko *et al.*, 2002; Banwat *et al.*, 2003; Cama *et al.*, 2003; Neira-Otero *et al.*, 2005; Tumwine *et al.*, 2005; Cama *et al.*, 2007). In Enugu state, SE Nigeria, all HIV-positive adults with diarrhoea tested negative for *Cryptosporidium* infection (Nwokediuko *et al.*, 2002). Similarly, in Jos, Nigeria, undernourished HIV-positive children were found to be free of infection while 3.8% of HIV-negative children were infected (Banwat *et al.*, 2003). In contrast to this low prevalence, Tumwine *et al.* (2005) reported a prevalence of 73.6% in HIV-positive children and 5.9% in HIV-negative children in Uganda (Table 1.5).

Chen *et al.* (2002), indicated that the rate of *Cryptosporidium* infection in people suffering from AIDS and diarrhoea in developed countries is about 14% (6-70%) while in developing countries it is about 24% (8.7-48%), whereas, Hunter and Nichols (2002), state that the prevalence of *Cryptosporidium* infection in HIV-positive patients with diarrhoea ranges from 0-100% with a median of 32%. Although data differs on the prevalence of infection in HIV-positive individuals, it is generally accepted that infection is higher, and poses a greater threat, to HIV-positive patients in comparison with HIV-negative individuals. This is due to the persistent and life threatening diarrhoea which occurs in patients with a CD4+ T cell count below 150/ml (Tzipori and Ward, 2002). As discussed in Section 1.4.4, the most effective treatment for *Cryptosporidium* infection is the use of HAART which increases the CD4+ T cell count in the immune system. However, in developing countries this treatment is often unavailable and so infection in patients in these regions can be more severe.

1.7.4 Age and infection

Although *Cryptosporidium* infections have been reported in humans ranging from 3 days old to 95 years, data imply that young children are most susceptible to infection (Fayer, 2004). Studies from developing countries suggest that most *Cryptosporidium* infection occurs in children <5 years old (Ludin *et al.*, 1991; Nimri and Hijazi, 1994; Bhattacharya *et al.*, 1997; Chacin-Bonilla *et al.*, 1997;

Iqbal *et al.*, 1999; Newman *et al.*, 1999; Abdel-Messih *et al.*, 2005). Within this age bracket, additional studies from Gambia, Malawi, Nigeria, Egypt, Bangladesh and Indonesia have indicated that children <2 years are most frequently infected (Adegbola *et al.*, 1994; Katsumata *et al.*, 1998; Nwabuisi, 2001; Khan *et al.*, 2004; Abdel-Messih *et al.*, 2005; Morse *et al.*, 2007). Abdel-Messih *et al.* (2005) found that children < 1 year old are 2.4 times more likely to be infected than older children (up to 5 years) and children aged 12-23 months are 1.9 times more likely to be infected than 24-36 month old children. However, other studies have found that age is not linked to prevalence of infection (Das *et al.*, 1993; Chacin-Bonilla *et al.*, 1997; Esteban *et al.*, 1998; Sulaiman *et al.*, 2005; Ajjampur *et al.*, 2007). Some reviews have indicated that children under the age of 6 months show low infection rates particularly in those children that are breast-fed (Nichols, 2008). As discussed by Nichols (2008), this may be as a result of the protective effect of breast-feeding, or from limited contact with potentially infective surfaces prior to walking.

It has been suggested that the diarrhoeal disease which results from infection with *Cryptosporidium* can have long-term negative effects on a child's growth and cognitive development (Molbak *et al.*, 1997; Checkley *et al.*, 1998; Guerrant *et al.*, 1999; Niehaus *et al.*, 2002). In Northeast Brazil, children suffered reduced fitness 4-6 years following early childhood diarrhoea, and specifically with cryptosporidial infections in the first 2 years of life (Guerrant *et al.*, 1999). This result was found independently of respiratory illness, anthropometry, anaemia and intestinal helminths. In addition, previous epidemiological studies have demonstrated a positive association between *Cryptosporidium* infection and malnutrition (Macfarlane and Horner-Bryce, 1987; Sallon *et al.*, 1988; Sarabia-Arce *et al.*, 1990; Checkley *et al.*, 1997; Checkley *et al.*, 1998; Tzipori and Ward, 2002). In a study carried out in Peru, it was demonstrated that *Cryptosporidium* infection, whether symptomatic or asymptomatic, had a long-lasting adverse effect on weight gain and linear growth (height) (Checkley *et al.*, 1998). The Peruvian population exhibits a high degree of nutritional stunting: 37% of children <5 years are stunted and Checkley *et al.* (1998) suggested that the effects of *Cryptosporidium* infection on growth are likely to be contributing to the prevalence of stunting in Peru. Furthermore, the authors point out that the magnitude and persistence of the effect is intensified for children with poorer nutritional status. This is consistent with results from Uganda (Tumwine *et al.*, 2005) where the authors found a significant association between *Cryptosporidium* infection and stunting, being underweight and wasting.

The species of *Cryptosporidium* which infected the children in the above mentioned studies on malnutrition were not determined, however, Bushen *et al.* (2007) indicated that height-for-age (HAZ) z-scores decreased for children infected for 3 months with either *C. hominis* or *C. parvum*. However, 3-6 months following infection only *C. hominis* infected children continued to show a decrease in height-for-age indicating differences between the species. According to Cordova Paz Soldon *et al.* (2006), infantile cryptosporidiosis is especially grave in children who are immunodepressed by malnutrition and it is important to establish prevention and control programmes against *Cryptosporidium*. It is not possible, however to determine whether malnutrition is caused by the infection through malabsorption along the digestive tract or whether children are predisposed to infection when malnourished during which time the immune system is compromised.

1.7.5 Gender and infection

Studies conducted in many countries (e.g. Malawi, Kenya, Gambia, Zambia, Egypt, Indonesia, India, Bolivia, Mexico and Jordan) have found no association between infection with *Cryptosporidium* and gender (Adegbola *et al.*, 1994; Miller *et al.*, 1994; Nimri and Hijazi, 1994; Bhattacharya *et al.*, 1997; Esteban *et al.*, 1998; Katsumata *et al.*, 1998; Nchito *et al.*, 1998; Newman *et al.*, 1999; Khan *et al.*, 2004; Abdel-Messih *et al.*, 2005; Gatei *et al.*, 2006a; Morse *et al.*, 2007). In addition, Ajjampur *et al.* (2007) determined the species of *Cryptosporidium* present in a paediatric population and also found no association with gender of the child between *C. hominis*-infected children and those infected with other species. In contrast, male children in Brazil were found to be 2.2 times more likely to be infected than females (Pereira *et al.*, 2002), while studies in Nigeria and Guatemala found that females had a higher prevalence of *Cryptosporidium* infection than males (Kwaga *et al.*, 1988; Okafor and Okunji, 1994; Laubach *et al.*, 2004) (Table 1.8).

1.7.6 Risk factors

Although risk factors for infection have been explored in both the developed and developing world, the majority have been conducted in developed countries with studies generally focusing on risk factors for outbreaks of disease rather than on

sporadic infection. Recently, studies have indicated that risk factors may differ for *C. hominis* and *C. parvum*, indicating that if species data are not included in risk factor analysis then the analysis may highlight risk factors common to both species but downplay risk factors which are important for 1 species or the other (Hunter *et al.*, 2004; Lake *et al.*, 2007). However, other studies have found no such variation in risk factors by species (Ajjampur *et al.*, 2007; Cama *et al.*, 2008) and so this area of research requires further investigation. Risk factors for sporadic infection in developed countries, are presented in Appendix 1 (Table A1.1) and include studies which have determined risk factors separately for *C. hominis* and *C. parvum*. Risk factors, protective (negative) factors and factors which had no association with infection are highlighted. In addition, Table 1.8 presents risk factors for sporadic infection in developing countries. As risk factors in developed and developing countries are likely to differ, more data is required from developing regions, in order to assess risk effectively.

Table 1.8 Risk factors for sporadic *Cryptosporidium* infection in developing countries

Location	Age group	Sample size (infected)	Risk factors	No Association	Reference
Texas-Mexico border	6 mo – 13yrs	285 (196)	Urban living Older age consumption of municipal water Lower annual household income	Water source Maternal age	Leach *et al.* (2000)
Mexico	< 5 yrs	403 (26)	Malnutrition Not breast-fed	Age Unboiled water Animal exposure Dwelling	Javier-Enriquez *et al.* (1997)
Guatemala	2-13 yrs	100 (32)	Females	Age	Laubach *et al.* (2004)
Brazil	2 wks-10yrs	445 (64)	Younger age Male Number of children in daycare Children in the household with diarrhoea Dwelling distance from water body (further = more risk)	Time in daycare Parents job No. of caregivers Breast-feeding Diet, food hygiene Drinking water Sewage presence Fruit & vegetables Animal exposure	Periera *et al.* (2002)
NE Brazil	0 – 4 yrs	189 (58)	Low birth weight Living in densely crowded areas	Water usage Gender Breast-feeding Malnutrition Pet exposure Maternal education	Newman *et al.* (1999)

Table 1.8 Contd

Location	Age group	Sample size (infected)	Risk factors	No Association	Reference
India	<5 yrs	46 cases (46 age-matched controls)	< 2 yrs	Animal exposure Water supply Food preparation Diarrhoeal history Gender Breast feeding Malnutrition	Khan *et al.* (2004)
India	0-59 months	68 cases (204 controls)	< 2 yrs Stunting Not breast-fed	Household income Underweight	Bhattacharya *et al.* (1997)
Indonesia	Children & adults	4368 (49)	<2 yrs of age Contact with cats Contact with flood Crowded living conditions	Gender Drinking water Public bathing Rooms/house	Katsumata *et al.* (1998)
Egypt	< 5 yrs	1275 (214)	Younger age Not breast-feeding	Gender	Abel-Messiah *et al.* (2005)
Guinea-Bissau	Children	125 cases 125 controls	Keeping pigs and dogs in the household Storage of cooked food for later consumption Male child Not breast-fed	Unknown	Molbak *et al.* (1994)

Table 1.8 Contd

Location	Age group	Sample size (infected)	Risk factors	No Association	Reference
Kenya	<5 yrs	4,899 (183)	13-24 months	Gender	Gatei *et al.* (2006a)
Zambia	<11 yrs	222 (39)	Living in areas with high-risk piped water Own their own house Not breast-fed	Age Gender Underweight Animal exposure Parent education	Nchito *et al.* (1998)

1.8 Aims

Various epidemiological studies based in developing countries have focused on prevalence and temporal variability of *Cryptosporidium* infection. However, few have determined risk factors for infection and less still have characterised the species, genotypes and subtypes present. Studies rarely take a holistic approach, assessing all of these factors in the same population.

Owing to the need for epidemiological data regarding high-risk paediatric populations in Nigeria and the general lack of molecular data in developing countries, the present study aims to:

1. Determine the prevalence and temporal variability of *Cryptosporidium* infection in a high-risk paediatric population in Nigeria

2. Identify the species, genotypes and subtypes present

3. Establish possible risk factors for infection in the population.

Molecular data, coupled with risk factors for infection will greatly add to our knowledge of the epidemiology of *Cryptosporidium* in West Africa.

2 Prevalence and temporal variability of *Cryptosporidium* infection in a Nigerian paediatric population

Section **Page**

Figure **Page**

Table **Page**

Plate **Page**

2.1 Introduction

Cryptosporidium is an Apicomplexan, protozoan parasite and is the causative agent of the diarrhoeal disease cryptosporidiosis. Although it causes acute short-term infection in immunocompetent patients, the disease can be severe in patients with a compromised immune system (especially the malnourished and those with HIV/AIDS) leading to chronic dehydration and even to death (Dillingham *et al.*, 2002).

Recently, attention has focused on childhood disease and studies have indicated that children are at a high risk of infection with those less than 5 years of age being most susceptible (Nimri and Hijazi, 1994; Bhattacharya *et al.*, 1997; Chacin-Bonilla *et al.*, 1997; Iqbal *et al.*, 1999; Newman *et al.*, 1999; Abdel-Messih *et al.*, 2005). It has been suggested that the diarrhoeal disease these young children suffer from as a consequence of infection can have long-term negative effects on their growth and cognitive development (Molbak *et al.*, 1997; Guerrant *et al.*, 1999; Niehaus *et al.*, 2002). Therefore, identifying the prevalence of infection from specific geographical regions is essential in order to determine the public health significance of infection in various locations. High-risk areas can then be identified and control measures to prevent transmission implemented.

Prevalence of childhood infections in developing countries such as Kenya, Nigeria, Peru, Chile, Bolivia, Bangladesh, Mexico and Gambia ranges from 1% - 31.6% (Adegbola *et al.*, 1994; Okafor and Okunji, 1996; Bhattacharya *et al.*, 1997; Javier Enriquez *et al.*, 1997; Esteban *et al.*, 1998; Neira-Otero *et al.*, 2005; Cordova Paz Soldon *et al.*, 2006; Gatei *et al.*, 2006a) and studies indicate that prevalence varies not only geographically, with increased prevalence in developing countries, but also temporally, with higher rates reported during the rainy season in many tropical countries (Adegbola *et al.*, 1994; Javier Enriquez *et al.*, 1997; Newman *et al.*, 1999; Pereira *et al.*, 2002; Peng *et al.*, 2003b; Das *et al.*, 2006; Morse *et al.*, 2007). In Nigeria, the most populous African country, cohort studies on the prevalence and intensity of *Cryptosporidium* infection from high-risk paediatric populations is lacking.

Therefore, this chapter addresses the following specific aims:

1. To determine the prevalence, temporal variability and intensity of *Cryptosporidium*

infection in a high-risk paediatric population at 4 time points over a 1-year period

2. To determine whether age, gender or village of residence of the child are associated with *Cryptosporidium* infection in this population.

2.2 Materials and Methods

2.2.1 Nigeria

This study was conducted in Osun state, Nigeria (Plate 2.1). Nigeria covers a land area of 923.8 sq km and has a population of 146.3 million. The annual population growth rate is 2.4% (World Bank statistics, 2007) and the Nigerian people account for 20% of the population of sub-Saharan Africa (Chapin Metz, 1991). Nigeria is typical of many developing countries in that the life expectancy at birth (47 years) is much lower than that of developed countries and the mortality rate for children under the age of 5 years is high, at 99/1000 live births. From 2000-03, the top 5 causes of death in children under the age of 5 years were neonatal causes, malaria, pneumonia, diarrhoeal diseases and measles (WHO, 2006). In the general population HIV/AIDS was the main cause of death followed by malaria, lower respiratory infection, diarrhoeal diseases, measles and TB (WHO, 2006). In 2007, 27% of children under the age of 5 were malnourished (underweight) (World Bank statistics, 2007). The prevalence of HIV is low relative to other sub-Saharan countries at 3.1% in 15-49 year olds (WHO, 2006).

2.2.2 Study site and population

Osun State is located in the south western region of Nigeria. It covers an area of approximately 14,875 sq km and is bounded by Ogun, Kwara, Oyo and Ondo states in the south, north, west and east, respectively (Plate 2.2).

The present study was carried out in 4 semi-urban villages, Akinlala (altitude 223m), Edunabon (234m), Ipetumodu (226m) and Moro (227m), surrounding the town of Ile-Ife, in the south-west of Osun state. Ipetumodu, Edunabon and Moro are all located within 2km of each other, while Akinlalu is located 6km south-west of the other 3 villages. All villages are 12-15 km west of the town of Ile-Ife (Plate 2.3) and fall under the catchment area of the Ife North Local Government (INLG) (985 sq km).

This area is characterised by a tropical climate, with the rainy season extending from April to October [with a dip in precipitation during August which is useful agriculturally as it allows for grain harvesting (Chapin Metz, 1991)] and the dry season from November to March. The rainfall is marked by a dual maxima (July and September) and a dual minima (August and January). Annual rainfall in the region ranges from 1000 to 4000 mm (Asaolu *et al.*, 1991) with a median of 1250 mm (Chapin Metz, 1991). The average maximum and minimum daily temperatures are 31 and 23°C in January and 28 and 23°C in June, repectively (Chapin Metz, 1991). The vegetation is predominately rainforest (Asaolu *et al.*, 1991).

A census undertaken in the Ife North Local Government area in 1991 estimated the population size to be 129,996. The people of this area are mostly Christians (>50%), Muslims and Afrelists i.e. adherents of Africa Traditional Religion (ATR), and are a mix of various ethnic groups with the majority Yoruba-speaking (although English is the official language in the country).

The houses in the villages are predominantly built of concrete walls and floors and roofed with galvanised iron sheets (Plate 2.4). There is no organised sewage disposal system and refuse and human faeces are dumped in the bush behind the houses or burned (Asaolu *et al.*, 1991). Goats, dogs and chickens are commonly seen wandering freely around the villages. Cattle are not normally reared in southern Nigeria. Most farmers in the area are peasant farmers. Many have small cocoa plantations and some plant cassava, corn, tomato, oranges but none is on extensive scale. The main sources of water are from shared community taps and/or wells located in each village. Health care is provided in health centres located in each village. However, these are inadequately equipped and lack qualified staff. The nearest hospital is located at Ile-Ife.

Plate 2.1 Location of Nigeria in sub-Saharan Africa
(Source: www.freeworldmaps.net/africa/index.html)

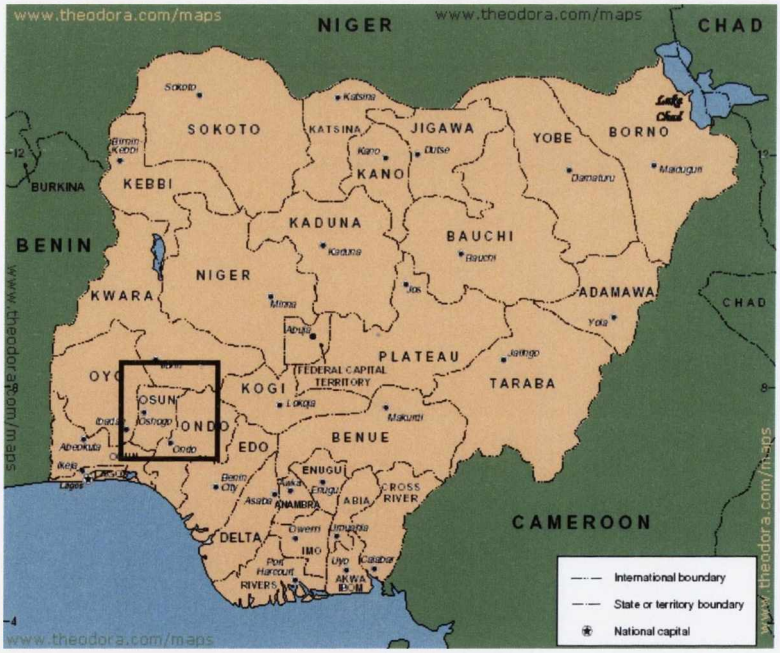

Plate 2.2 Location of Osun state in south west Nigeria
(Source:www.theodora.com/maps/new8/nigeria_divisions.gif)

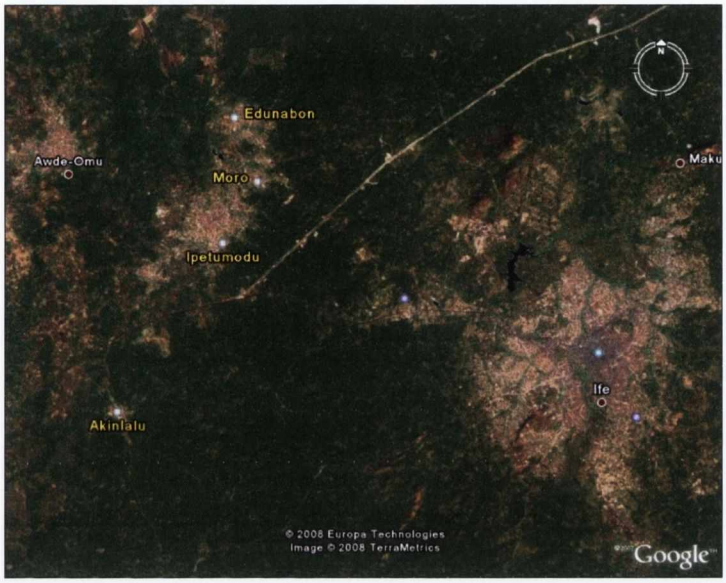

Plate 2.3 Location of Akinlalu, Ipetumodu, Moro, Edunabon and Ile-Ife
The distance from Ipetumodu to Moro is 2km (Source: Google Earth)

Plate 2.4 Typical village house with concrete walls and corregated iron roof
(Photo by: Síle Molloy)

2.2.3 Stool sample collection

This study was part of a parallel project which began in May 2006 and assessed the prevalence of malaria and *Ascaris* infection in the same cohort of children (Kirwan *et al.*, 2009). At this time meetings were held with INLG and the Obas of each village to gain their support for the study and to explain the aims (i.e. to look at the interaction between worms and malaria in children aged 1-6 years). The Oba is the traditional head of a Yoruba village. Introductory meetings were then held in each village to explain the purpose of the study to the women of the villages and the people who attended were asked to disseminate this information to the rest of the community. The Obas made a call for children aged 12-60 months to attend temporary clinics for assessments on specified dates. A total of 2332 children, aged between 11 months and 6 years, were enrolled in the study during May and September 2006, following informed consent. Malaria nets were given to study participants at the end of the study and children were given multi-vitamins at each time point as an incentive. Temporary clinics took place in the centre of each village and mothers from the surrounding area were asked to bring their children for screening of malaria and intestinal worms. Once enrolled in the programme, each child was assigned an ID number. In order to assess the temporal variability of infection, clinics were run at 4 time points over a 1 year period: September 2006, February 2007, May 2007 and August 2007. These time points included both the rainy (April – October) and the dry (November – March) seasons. Anthropometric data (heights and weights) were also taken in August 2007. At each time point, mothers and children were invited to attend the clinics and mothers were supplied with 50 ml plastic containers in which to collect their children's faeces (Plate 2.5). Faecal samples were returned to the clinic, transported to the laboratory in a cool box and refrigerated at 4°C. Results of malaria infection was given to the mothers on the day of assessment and worm and *Cryptosporidium* infection data was given at the end of the study in August 2007.

Of the 2332 children enrolled, 1636 children submitted faecal samples on at least 1 of the 4 time points. A number of children were lost to follow-up with 349 children submitting samples at each of the 4 time points. Ethical clearance was granted by the Ethics and Research Committee of Obafemi Awolowo University Teaching Hospital Complex, Ile-Ife, Nigeria.

Plate 2.5 Mothers supplied with 50ml container to collect child's stool sample (Photo: Síle Molloy)

2.2.4 Analysis of stool samples

Samples were transported to Ireland unfixed at the end of each time point and processed immediately. Samples were processed separately to determine the prevalence of *Ascaris* infection and *Cryptosporidium* infection. Processing took place at the same time and in the same laboratory but by separate researchers. For the determination of *Cryptosporidium* infection, a portion of each sample was processed (with the remaining sample refrigerated for future molecular work) using the modified formal–ether concentration technique of Allen and Ridley (1970), and auramine-phenol staining (Casemore *et al.*, 1985; Fleck and Moody, 1988). One slide per child was prepared. Slides were viewed under a fluorescent microscope to detect the presence of oocysts.

2.2.4.1 Formal-ether concentration

Stool consistency was evaluated by visual examination of the stool on the same day as stool collection and classified as formed, unformed or watery. A pea-sized amount (200 µL if liquid) of faeces from each child was concentrated using a modified version of the formol-ether technique developed by Allen and Ridley (1970) (Casemore *et al.*, 1985). This technique concentrates the oocysts from the faeces (by centrifugation) while removing particulate matter from the sample (by sieving) which may mask the

presence of oocysts on the slide. The sensitivity of the diagnostic method is thus increased through concentration.

As described by Smith (2008), a pea-sized amount of faeces (approx. 750 µL if liquid stool) was placed in a clean 15 ml graduated centrifuge tube using an applicator stick and the sample was thoroughly emulsified in 7 mls of 10% formalin. The suspension was then filtered through a 425 µm aperture sieve to remove large particles and the filtrate poured back into the same tube. Any debris caught in the sieve was discarded. 3 mls of the solvent di-ethyl ether was added to the filtrate (to remove fats and oils) and shaken vigorously for 30 s. The tube was centrifuged at 1200 x g for 2 min. After centrifugation, the supernatant (with a fatty plug) was decanted, leaving 1-2 drops with the remaining pellet. The pellet was resuspened by agitation, transferred onto a glass slide and air dried. The slides were then stained using the auramine–phenol method (Casemore *et al.*, 1985; Fleck and Moody, 1988).

2.2.4.2 Auramine-phenol staining

This method uses the flourogenic stain auramine-phenol (AP) and the counter stain, potassium permanganate (Casemore *et al.*, 1985; Fleck and Moody, 1988). Auramine-phenol staining (AP) was shown to be more sensitive than the modified Ziehl Neelson method (mNZ) (Casemore *et al.*, 1985) as it stains both internal structures and the outer walls (Smith, 2008). In addition, Morse *et al.* (2007) showed that AP staining was more sensitive than both mZN staining and immunoflourescence (IF). Using AP stain 40/50 positive samples were identified, however, IF identified only 36/50 samples and mZN 31/50 samples. Although Webster *et al.* (1996) found that IMS was more sensitive than AP stain for the detection of oocysts, cost restraints prevented the use of IMS in the current study. Thus, AP staining was used.

Slides with air dried faecal smears were placed in multi-slide carriers and stained in batches. As described by Smith (2008), the slides were placed in a trough of methanol for 10 min and then immersed in AP stain for 10 min. Slides were rinsed in tap water to remove excess stain and decolourised in 3% HCL in methylated alcohol for 5 min. They were then rinsed in tap water and counterstained in 0.1% potassium permanganate for 30 s. Slides were re-rinsed in tap water and air-dried in preparation for microscopic examination. AP staining allows the oocysts to be seen

with the flourescein isothiocyanate (FITC) filter and UV filters of an epiflourescent microscope.

2.2.4.3 Microscopy

Slides were examined by the one person for the presence of oocysts using a fluorescent microscope (blue filter block; excitation 490 nm, emission 510 nm). Each slide was scanned at 200x magnification (field of view, 1800 µm) and oocysts were confirmed under 400x magnification (field of view, 450 µm). The first 100 slides were double checked by another researcher to ensure that the results were accurate. *Cryptosporidium* oocysts appear doughnut-shaped and fluoresce brightly against a dark background (Plate 2.6). Positive and negative samples were recorded along with the intensity of infection. Intensity was determined as follows: 0 = no oocysts detected in the sample; 1+ = 1-10 oocysts per field of view; 2+ = 10-50 oocysts per field of view and 3+ = >50 oocysts per field of view. The number of oocysts/ml was not calculated.

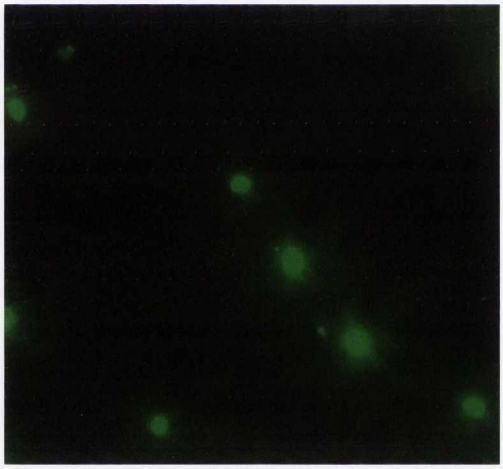

Plate 2.6 *Cryptosporidium* oocysts under a fluorescent microscope (400x magnification) (Photo: Síle Molloy)

2.2.5 Statistical analysis

Chi-squared analysis ($\chi^2 = \Sigma$ (0bserved frequency – Expected frequency)^2 / Expected frequency) was performed to test for associations between oocyst presence and stool consistency, oocyst intensity (1+ versus 2+ and 3+ combined) and stool consistency,

and to test for associations between age, gender and village of residence, and infection status of the children within each of the 4 months.

Of the 1636 children assessed in the study, 349 submitted samples at all 4 time points. A generalized linear latent and mixed model (gllamm) analysis was performed in STATA, Version 10.0 (Rabe-Hesketh *et al.*, 2002) on the infection status of these 349 children. The model was used to test for associations between month, age, gender and village of residence, and infection status of the children. This model incorporated both random and fixed variables accounting for repeated measures in the longitudinal data. Tracking the same children throughout the year and comparing prevalence data at each of the 4 time points represents a more powerful data set owing to the reduced influence of host variability on the observed epidemiological patterns. In addition, the statistical model employed for analysis is numerically stable when there are few missing values.

2.3 Results

A total of 3840 samples from 1636 children were examined over the 1 year period. For those children where gender data were available, 825 (50.4%) were male and 790 (48.3%) female. Children were aged between 6 and 80 months, with a median of 39 months. A total of 266 children (16.3%) were from the village of Akinlalu, 714 (43.6%) from Ipetumodu, 253 (15.5%) from Moro and 403 (24.6%) from Edunabon.

The overall prevalence of *Cryptosporidium* infection, determined by the detection of *Cryptosporidium* oocysts in the first sample submitted from each child, was 15.2% (248/1636, 95% CI: 13.27-16.73%). In total, 684/3840 samples, 17.8% (95% CI: 16.61-19.06%) were positive for *Cryptosporidium* oocysts (Table 2.1). The prevalence of infection ranged from 15.6% (95% CI: 13.17-18.16%) in September 2006 to 19.6% (95% CI: 17.31-22.18%) in May 2007 (Fig 2.1 A, Table 2.1). Of the 1636 children that submitted stool samples, 579 (35.4%) children were infected on at least 1 occasion over the 1 year period.

Table 2.1 Prevalence of *Cryptosporidium* infection in Nigerian children at 4 time points over 1 year

Infection Status	Month (%)				Total
	Sept	Feb	May	Aug	
Infected	132 (15.6)	206 (17.3)	209 (19.6)	137 (18.6)	684 (17.8)
Uninfected	717 (84.4%)	987 (82.7)	854 (80.4)	598 (81.4)	3156 (82.2)
Total	849 (100)	1193 (100)	1063 (100)	735 (100)	3840 (100)

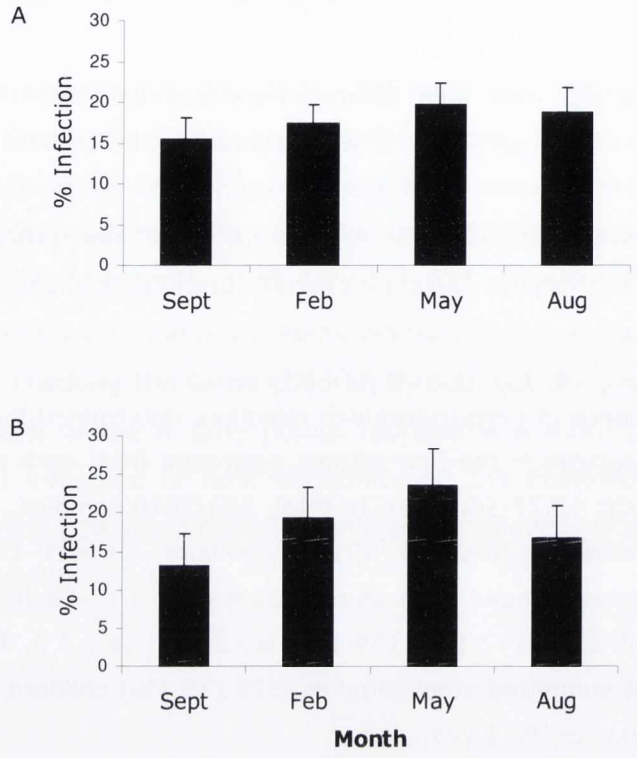

Figure 2.1 Prevalence (± 95% CI) of *Cryptosporidium* infection in Nigerian children at 4 time points over 1 year. (A) All samples collected (B) Samples from 349 children that attended each of the 4 time points.

The majority of samples (652 of 684) had an oocyst intensity of 1+ (1-10 oocysts per field of view). A total of 21 children had an oocyst intensity level of 3+ (>50 oocysts per field of view). Of these, 11 occurred in August in comparison with 1 in September/October, 0 in February and 9 in May (Table 2.2).

Table 2.2 Intensity of *Cryptosporidium* infection in all stool samples collected at each of the 4 time points

	Month (%)				
Intensity	**Sept**	**Feb**	**May**	**Aug**	**Total (%)**
1+	132 (99.3)	206 (100)	194 (93.3)	122 (89.1)	654 (95.7)
2+	0	0	5 (2.4)	4 (2.9)	9 (9.0)
3+	1 (0.7)	0	9 (4.3)	11 (8.0)	21 (3.3)

There was no statistical association between the presence of oocysts and stool consistency in any of the 4 months studied (Table 2.3), nor between oocyst intensity and stool consistency (Table 2.4).

Table 2.3 Stool consistency and infection status at each of the 4 time points

	Stool consistency (%)					
Month	**Formed**	**Unformed**	**Watery**	χ^2	**df**	**p value**
September						
Infected	61 (46.2)	65 (49.2)	6 (4.6)	3.512	3	0.173
Uninfected	272 (38.0)	394 (55.1)	49 (6.9)			
Total	333 (36.8)	459 (54.2)	55 (6.5)			
February						
Infected	76 (36.9)	116 (56.3)	14 (6.8)	0.0005	3	1.0
Uninfected	363 (36.8)	556 (54.6)	67 (6.8)			
Total	439 (36.8)	672 (56.4)	81 (6.8)			
May						
Infected	72 (34.5)	124 (59.3)	12 (6.2)	2.330	3	0.312
Uninfected	249 (29.2)	542 (63.5)	63 (7.4)			
Total	321 (30.2)	666 (62.6)	76 (7.2)			
August						
Infected	36 (26.3)	94 (68.6)	7 (5.1)	4.935	3	0.085
Uninfected	184 (30.8)	402 (67.2)	12 (2.01)			
Total	220 (29.9)	496 (67.5)	19 (2.6)			

Table 2.4 Association between stool consistency and oocyst intensity

	Stool consistency (%)					
Intensity	**Formed**	**Unformed**	**Watery**	χ^2	**df**	**p value**
1+	237 (36.3)	377 (57.8)	38 (5.8)	2.21	2	0.346
2+ & 3+	7 (11.1)	21 (70)	2 (6.7)			
Total	244 (35.8)	398 (58.4)	40 (5.7)			

September 2006

Of the 862 samples analysed in September 2006, the total prevalence (± 95% CI) of *Cryptosporidium* infection was 15.6% (13.17 – 18.16) with child age ranging from 11 to 77 months. There was no statistically significant association between prevalence of *Cryptosporidium* infection and gender, age or village of residence (Table 2.5).

February 2007

Of the 1203 samples analysed in February 2007, the total prevalence (± 95% CI) of *Cryptosporidium* infection was 17.0% (14.9 – 19.2) with child age ranging from 15 to 79 months (Table 2.6). Although there was no statistically significant association between prevalence of *Cryptosporidium* infection and gender, age or village of residence, there was a trend present for an association between *Cryptosporidium* infection and gender ($p=0.062$), with males more likely to be infected than females.

May 2007

Of the 1073 samples analysed in May 2007, the total prevalence (± 95% CI) of *Cryptosporidium* infection was 19.6% (17.2 – 21.9) with child age ranging from 18 to 76 months. There was no statistically significant association between prevalence of *Cryptosporidium* infection and gender, age or village of residence (Table 2.7).

August 2007

Of the 735 samples analysed in August 2007, the total prevalence (± 95% CI) of *Cryptosporidium* infection was 19.0% (16.2 – 21.9) with child age ranging from 21 to 80 months. There was no statistically significant association between prevalence of *Cryptosporidium* infection and gender, age or village of residence (Table 2.8).

Table 2.5 The relationship between *Cryptosporidium* infection and gender, age and village of residence in September 2006

Variable	Prevalence of infection (%)	χ^2 value	df	p value
Gender				
Male (n=435)	17.5			
Female (n=403)	13.4	2.646	1	0.104
Total (n= 838)*	15.8			
Age (months)				
11-23.5 (n=264)	14.0			
24-35.5 (n=201)	15.9			
36-47.5 (n=188)	15.4	1.039	4	0.904
48-59.5 (n=136)	17.6			
>60 (n=59)	17.0			
Total (n=848)**	15.8			
Village				
Akinlau (n=191)	19.9			
Ipetumodu (n=288)	12.2			
Moro (n=140)	17.9	5.95	3	0.114
Edunabon (n=230)	14.8			
Total (n=849)***	15.8			

*Missing data for gender: 24

**Missing data for age: 14

***Missing data for village: 13

Table 2.6 The relationship between *Cryptosporidium* infection and gender, age and village of residence in February 2007

Variable	Prevalence of infection (%)	χ^2	df	p value
Gender				
Male (n=608)	18.9	3.491	1	0.062
Female (n=573)	14.8			
Total (n= 1181)*	16.9			
Age (months)				
11-23.5 (n=228)	20.2			
24-35.5 (n=341)	16.4			
36-47.5 (n=232)	16.0	3.49	3	0.322
48-59.5 (n=234)	15.8			
>60 (n=156)	17.3			
Total (n=1191)**	17.0			
Village				
Akinlau (n=190)	15.8			
Ipetumodu (n=547)	15.9	2.13	4	0.712
Moro (n=181)	16.0			
Edunabon (n=275)	20.7			
Total (n=1193)***	17.0			

*Missing data for gender: 22

**Missing data for age: 12

***Missing data for village: 10

Table 2.7 The relationship between *Cryptosporidium* infection and gender, age and village of residence in May 2007

Variable	Prevalence of infection (%)	χ^2	df	p value
Gender				
Male (n=535)	20.0			
Female (n=516)	19.0	0.170	1	0.680
Total (n= 1051)*	19.5			
Age (months)				
11-23.5 (n=127)	22.8			
24-35.5 (n=304)	19.1			
36-47.5 (n=212)	19.3	1.361	4	0.851
48-59.5 (n=233)	20.2			
>60 (n=186)	17.7			
Total (n=1062)**	19.6			
Village				
Akinlau (n=190)	16.8			
Ipetumodu (n=476)	20.4			
Moro (n=149)	20.8	1.248	3	0.742
Edunabon (n=248)	19.4			
Total (n=1063)***	19.6			

*Missing data for gender: 22

**Missing data for age: 11

***Missing data for village: 10

Table 2.8 The relationship between *Cryptosporidium* infection and gender, age and village of residence in August 2007

Variable	Prevalence of infection (%)	χ^2	df	p value
Gender				
Male (n=378)	17.5			
Female (n=348)	20.1	o.838	1	0.36
Total (n= 726)*	19.0			
Age (months)				
11-23.5 (n=30)	13.3			
24-35.5 (n=215)	19.5			
36-47.5 (n=165)	17.0	1.543	4	0.891
48-59.5 (n=163)	20.9			
>60 (n=161)	18.0			
Total (n=734)**	19.0			
Village				
Akinlau (n=151)	13.9			
Ipetumodu (n=319)	17.9			
Moro (n=100)	21.0	4.82	3	0.185
Edunabon (n=165)	23.0			
Total (n=735)	19.0			

*Missing data for gender: 9

**Missing data for age: 1

Of the 1636 children that submitted faecal samples for analysis, 502 children came at 1 time point, 411 came twice, 374 came 3 times and 349 children submitted samples at each of the 4 time points. Of these 349 children, 180 (51.6%) were male and 165 (47.3%) female (4 children had missing data for gender). Children were aged between 11 and 77 months, with a median of 39 months. Ninety-five children (27.22%) were from the village of Akinlalu, 119 (34.1%) from Ipetumodu, 56 (16.1%) from Moro and 79 (22.6%) from Edunabon. Prevalence of *Cryptosporidium* infection ranged from 13.2% (95% CI: 9.81-17.19) in September 2006 to 23.5% (95% CI: 19.15-28.30) in May 2007 (Fig 2.1 B). The majority of these samples (238 of 252) had intensity levels of 1+ (1-10 oocysts per field of view). Altogether 10 children had an oocyst intensity level of 3+ (>50 oocysts per field of view). Of the samples with a 3+ intensity, a higher number occurred in August (8) in comparison with September (0), February (0) and May (2) (Table 2.9). Of these 349 children, 153 were never infected with *Cryptosporidium,* 148 were infected on 1 occasion, 43 were infected twice, 5 were infected 3 times and no child was infected on all 4 occasions. Not all children who were infected on more than 1 occassion were infected on consecutive occasions.

Table 2.9 Intensity of *Cryptosporidium* infection in 349 children under the age of 5 years in Nigeria who submitted faecal samples at each of the 4 time points over the course of 1 year

	Month (%)			
Intensity	**Sept**	**Feb**	**May**	**Aug**
1+	46 (100)	67 (100)	76 (93.9)	49 (84.7)
2+	0	0	3 (3.7)	1 (1.7)
3+	0	0	2 (2.4)	8 (13.6)

Using gllamm analysis (Rabe-Hesketh *et al.*, 2002), month was statistically significantly associated with infection, with increased risk in both May (OR 1.11, 95% CI: 1.049-1.178, $p < 0.0001$) and February 2007 (OR 1.06, 95% CI: 1.003-1.127, $p = 0.034$) in a model adjusted for age, gender and village of residence. There was no statistically significant association between infection status and age, gender or village of residence of the children (Table 2.10).

Table 2.10 Results from gllamm analysis testing for association between infection status and month, age, gender and village of residence of 349 children that submitted samples at 4 time points

Variable	Coeff.	Std. Err.	z	p >|z|	95% CI
Month					
September	Reference				
February	0.0621	0.029	2.12	0.034	0.005 – 0.120
May	0.106	0.030	3.59	0.000	0.048 – 0.164
August	0.040	0.030	1.34	0.179	-0.018 – 0.010
Age	-0.0002	0.001	-0.36	0.719	-0.002 – 0.001
Gender					
Male	Reference				
Female	-0.004	0.21	-0.21	0.834	-0.045 – 0.036
Village					
Moro	Reference				
Akinlalu	-0.031	0.033	-0.96	0.337	-0.096 – 0.033
Ipetumodu	-0.043	0.032	-1.37	0.172	-0.106 – 0.019
Edunabon	0.015	0.035	0.43	0.668	-0.053 – 0.083

2.4 Discussion

2.4.1 Prevalence

Our findings show that the overall prevalence of *Cryptosporidium* from a sample of Nigerian children ranged from 15.6% to 19.6% over a 1 year period. This finding is based on a large sample size of 3840. However, the study may have some potential limitations that need to be considered when interpreting the findings. It must be noted that this was not a truly random sample as children were self-selected i.e. mothers were asked to bring their children to the clinics for assessment. Furthermore, mother's enrolled their children into the programme primarily for the screening of malaria and worms which may also introduce a sample bias. Achieving a random sample of study participants in this field setting would prove very difficult owing to the widely dispersed nature of these semi-urban communities, and to the restricted age group being studied. Although a school-based study may have achieved a random sample it would have been impossible to target the age group of children required and sick children are likely to be absent from school, thus introducing a new bias to such a study. The large sample size assessed and the small age range incorporated in the current study is likely to compensate for the non-random selection of study participants.

Prevalence rates from developing countries range from 0-32% (mean of 34 studies = 11.8%) (see Chapter 1, Table 1.5) indicating that the prevalence of infection determined in the current study is relatively high in comparison with other studies. Of the few studies conducted to date in Nigeria, prevalence has ranged from 0%, in HIV-positive and HIV-negative patients with diarrhoea in Enugu state, to 39%, in primary school children with diarrhoea in the same state (mean of 7 surveys = 10.8%) (Reinthaler *et al.*, 1987; Kwaga *et al.*, 1988; Okafor and Okunji, 1994; Okafor and Okunji, 1996; Nwabuisi, 2001; Nwokediuko *et al.*, 2002; Okodua *et al.*, 2003). However, comparing epidemiological data on *Cryptosporidium* infection is difficult owing to variations in study design (longitudinal or cross-sectional), sample size, age-group studied, disease status of patients, location of the survey, the time of year survey was conducted and variations in laboratory techniques (which may have varying sensitivities of detection).

Focusing on the studies from Nigeria in order to highlight these variations, Okafor and Okunji (1994) recorded the prevalence of *Cryptosporidium* infection as 12.5%. However, this study incorporated both adults and children with diarrhoea and was a hospital-based study located in Nsukka, Nigeria in the south east of the country. In Abeokuta state, the prevalence was reported to be 0.9%, however the mean age of the patients examined was 32 years and the main focus of the study was to investigate prevalence of *Cryptosporidium* infection in HIV-negative (0%) and HIV-positive (5.7%) patients (Okodua *et al.*, 2003). Reinthaler *et al.* (1987) studied *Cryptosporidium* for 1 month over the rainy season in Ogun state, Nigeria (neighbouring state to Osun state). These authors collected samples from children with diarrhoea and found a prevalence of 2.3%, while Okafar and Okunji (1996) quoted a prevalence of 25.7% in children <15 years old with diarrhoea, in Enugu state (southern Nigeria). These variations in study design highlight the difficulties of comparing data and caution must be taken when making comparisons. Taking into account differences among the studies, our results indicate that the prevalence in this pediatric population in Nigeria is relatively high in comparison with other studies conducted in Nigeria and other developing countries (see Chapter 1, Table 1.5), especially as many studies have focused on children with diarrhoea which would potentially increase the prevalence of *Cryptosporidium* found in these areas.

2.4.2 Temporal variability and intensity

In the current study, prevalence was lowest in September and highest in May confirming the seasonality of infection in this area. Although children are getting older during the study this is not likely to influence the prevalence of infection and seasonality as it was shown that within each month the prevalence of infection did not differ between the age groups. The rainy season in this region of Nigeria occurs from April to October (with a break in the rains in August) and the dry season from November to March. Therefore, although there was a statistically significant difference in prevalence among the months, rainfall does not appear to be associated with infection rates. It is likely that the variation in prevalence over time is associated with practices or behaviours in the community which vary throughout the year. Previous studies have provided conflicting results in relation to seasonality of infection. Many have indicated that prevalence of infection is highest in months with the greatest rainfall (Newman *et al.*, 1999; Peng *et al.*, 2003b; Tumwine *et al.*, 2003; Morse *et al.*, 2007), however, Gatei *et al.* (2006a) found that in Kenya, prevalence was highest during the dry season (November –

February) that follows the short rains, while in Egypt and Venezuela no seasonal variation was observed (Chacin-Bonilla *et al.*, 1997; Abdel-Messih *et al.*, 2005).

Data on intensity of infection are generally lacking, particularly at a population level. The overall intensity of infection in this paediatric population was low with few oocysts observed in the samples. In August however, higher intensity levels were observed. The explanation for this low intensity is not clear although it may be linked to the apparently high levels of asymptomatic infections in these children (see Section 2.4.4). Not all children who were infected on more than 1 occasion were infected on consecutive occasions indicating that children were clearing the infection and becoming re-infected over time. Therefore, there appears to be a lack of immunity to infection in this population.

2.4.3 Demographic risk factors

Our study identified no statistically significant difference between *Cryptosporidium* infection and gender, age or village of residence of the child, either within each of the 4 months or in the 349 children which were followed over the full year. Previous studies from Indonesia, Egypt, Kenya, Zambia, India and Pakistan have also found no association between infection and gender (Das *et al.*, 1993; Katsumata *et al.*, 1998; Nchito *et al.*, 1998; Iqbal *et al.*, 1999; Abdel-Messih *et al.*, 2005; Gatei *et al.*, 2006a). In addition, Fayer and Ungar (1986) state that, based on large scale surveys reporting sex distribution, males and females appear to be equally susceptible to cryptosporidiosis. However, some studies have shown a difference in the susceptibility of males and females. In Brazil, males were 2.2 times more at risk of infection than females (Pereira *et al.*, 2002), while in Nigeria prevalence was higher in females than in males for all age groups of children aged between 2 and 15 years (Okafor and Okunji, 1994).

The age at which infection occurs can range from 3 days to 95 years (Fayer and Ungar, 1986), however, owing to the fact that more recent studies have indicated that children <5 years old are most susceptible (Ludin *et al.*, 1991; Nimri and Hijazi, 1994; Bhattacharya *et al.*, 1997; Chacin-Bonilla *et al.*, 1997; Iqbal *et al.*, 1999; Newman *et al.*, 1999; Abdel-Messih *et al.*, 2005) our study focused on infection within this age group. Until the age of 5 years, data vary on the age at which the child is most susceptible. Our study found no association between prevalence of infection and children separated into the following age groups: 1-2 years, 2-3 years, 3-4 years, 4-5 years and over 5 years. In contrast, many studies have found that children aged between 6 months and 2 years are most

susceptible. Pereira *et al.* (2002) found that infection is significantly greater in children <24 months old in comparison with older children. Likewise in Egypt, children aged <12 months were 2.4 times more likely to be infected as older children and those aged 12-23 months were 1.9 times more likely to be infected than older children (Abdel-Messih *et al.*, 2005). Iqbal *et al.* (1999) stated that prevalence in Pakistani children was highest in children aged 19-24 months in comparison with those aged <6 months. This is consistent with the findings of Pape *et al.* (1987) who found that children aged 6-24 months were twice as susceptible than children aged <6 months. Decreased risk for infection in children <6 months may be owing to the protective effect of breast-feeding in this age group or more likely owing to the lack of contact with the environment that these children experience prior to walking. However, the present study did not assess children under the age of 6 months and found no such relationship between older age groups and infection with *Cryptosporidium*.

There was no association between infection and the village of residence of the child, indicating that living each of the 4 villages pose similar risks for infection.

2.4.4 Asymptomatic infection

Although diarrhoeal status was not determined conclusively in this study (with diarrhoeal status in conjunction with the presence of oocysts in the stool assumed to be the major indicator of the presence or absence of cryptosporidiosis), it appears that there were a large number of asymptomatic cases present (based on a high number of infected individuals with formed stools (35.8%), a very low number of infected individuals with watery stools (5.7%), lack of an association between stool consistency and infection and a dominance of low intensity infections in the population). However, it must be noted that only 1 sample was taken from each child at all of the 4 time points and so a child with formed stools may not yet have experienced diarrhoea or may have had a recent history of diarrhoea and although the diarrhoea was resolved, the child may still have been shedding oocysts.

There is little information on the significance and importance of asymptomatic infection. A large number of studies have focused solely on patients with diarrhoea when assessing the prevalence of *Cryptosporidium* infection. Of those studies which assess the prevalence in both diarrhoeic and non-diarrhoeic cases [from

Chapter 1, Table 1.5, the prevalence of infection in adults and children without diarrhoea ranges from 0-31.6% (mean of 22 studies = 4.5%)] only a small number investigated and discussed the significance of these asymptomatic infections (Chacin-Bonilla *et al.*, 1993; Esteban *et al.*, 1998; Houpt *et al.*, 2005; Palit *et al.*, 2005; Siwila *et al.*, 2007). In Venezuala, *Cryptosporidium* infection was determined in 27 individuals and 15 were asymptomatic (Chacin-Bonilla *et al.*, 1993). Likewise, asymptomatic carriage was noted in dairy farm workers in Tanzania (6%) (Siwila *et al.*, 2007), in children from semi-urban slums in Kolkata, India (2.3%) (Palit *et al.*, 2005), in children and adults in Korea (3.3%) (Yu *et al.*, 2004) and a remarkably high level of asymptomatic infection was found in Bolivian children (31.6%) (Esteban *et al.*, 1998).

Although carriers do not suffer from the disease, asymptomatic infection has been shown to be associated with significant growth shortfall in children in Peru (Checkley *et al.*, 1997; Checkley *et al.*, 1998). Checkley *et al.* (1997) found that asymptomatic infection affected the growth of Peruvian children <2 years old and in a follow-up study (Checkley *et al.*, 1998) found a lasting adverse effect on the linear growth in children infected at an early age. Likewise, asymptomatic infection was associated with a shortfall in growth in children infected with *Giardia* (Prado *et al.*, 2005). The shortfall in growth was greater in children who remained free of diarrhoea indicating that infection can impede child growth even when asymptomatic, presumably through malabsorption. In addition, asymptomatic hosts are sources of infection for others in the community, many of whom may be more susceptible to infection (such as malnourished children and those with HIV/AIDS). The significance of asymptomatic infections may therefore be underappreciated, especially in developing countries where the prevalence of malnutrition (especially in young children) and HIV/AIDS may be high.

It is not clear why certain individuals exhibit a lack of clinical symptoms when infected with *Cryptosporidium*. As discussed by Esteban *et al.* (1998), it is possible that continuous exposure to infection promotes the development of immunity and therefore, re-infection results in mild or asymptomatic infection. Alternatively, the low levels of oocyst intensity, found in our study and similarly in a Tanzanian study (Houpt *et al.*, 2005), relative to that investigated in other studies (Goodgame *et al.*, 1993) could contribute to the apparent lack of symptoms.

2.4.5 Summary

Overall, *Cryptosporidium* infection in these Nigerian children was high (15.2%) relative to previous epidemiological studies and a number of children were infected on more than 1 occasion. However, as children were not always infected on consecutive occasions it appears that certain children cleared the infection and became re-infected over time, thus indicating a lack of acquired immunity. In addition, 35.4% of the 1636 children and 56.2% of the 349 children assessed on each of the 4 time points were infected on at least 1 occasion over the 1 year period, highlighting the high risk of infection for these children over a relatively short period of time.

Our findings indicate that the prevalence of infection varied over time and this observed seasonality was statistically significant when the same children were examined over the 1 year period. Observing the same children throughout the year allows for a reduced influence of host variability on the observed epidemiological patterns. The factors influencing seasonality of infection are unknown. Seasonal rainfall did not appear to contribute to the variation. It is possible that particular socio-economic or behaviour factors within the community are influencing the seasonality of infection. Thus, future work should aim to investigate behaviours in the population which may contribute to the temporal variability of infection.

Following this extensive base-line study on *Cryptosporidium* infection, a number of positive samples were chosen and the species, genotypes and subtypes of *Cryptosporidium* were determined in order to better understand the epidemiology of *Cryptosporidium* infection in this region of Nigeria.

3 Species, genotypes and subtypes of *Cryptosporidium* in a Nigerian paediatric population

Section **Page**

Figure **Page**

Table **Page**

Plate **Page**

3.1 Introduction

Understanding the epidemiology of *Cryptosporidium* infection has been a challenge facing researchers for decades. Difficulties have arisen owing to problems in defining the taxonomy of the parasite, identifying the multiple sources of infection and unravelling the various transmission routes. Defining the taxonomy of *Cryptosporidium* has been problematic as there was no clear definition of a species. Traditionally oocyst morphology, site of infection and host specificity were utilised as determinants of species identification, however, using these features proved difficult as most species possess morphologically indistinguishable oocysts. In addition, the sites of infection and host range overlap for a number of species. A new system was required for organising the taxonomy of *Cryptosporidium* and in recent years, molecular techniques have revolutionised this area of research. Molecular characterisation has established a genetic basis for defining species of *Cryptosporidium* and has revealed that the parasite exhibits a genetically diverse population structure (Mallon *et al.*, 2003).

Cryptosporidium parvum was once believed to infect over 155 mammalian hosts (Fayer, 2004). However, with the advance of molecular techniques, it has been delineated into a number of species and genotypes. At the close of the 20th century, molecular-based studies identified 2 types of *C. parvum*. Type 1, which was associated only with human infections, and Type 2, which was associated with infections in both humans and ruminants. In 2002, *C. parvum* Type 1 was elevated to species status and renamed *C. hominis* (Morgan *et al.*, 2002). Likewise, many more species have been validated and there are now 21 described species of *Cryptosporidium* of which 8 (*C. hominis*, *C. parvum*, *C. meleagridis*, *C. felis*, *C. canis*, *C. suis*, *C. muris* and *C. andersoni*) infect both immunocompetent and immunocompromised humans (Chalmers *et al.*, 2002; Jirků *et al.*, 2008; Fayer and Santin, 2009; Smith and Nichols, 2009a). *C. hominis* and *C. parvum* are the most frequently detected with *C. hominis* infections more common in developing countries (Morgan *et al.*, 2000a; Xiao *et al.*, 2001a; Cama *et al.*, 2003; Peng *et al.*, 2003b; Akiyoshi *et al.*, 2006; Gatei *et al.*, 2006a; Ajjampur *et al.*, 2007; Morse *et al.*, 2007). Both species have different host ranges. *C. hominis* is confined mostly to humans while *C. parvum* infects both humans and ruminants, with livestock considered a major reservoir for human infection (Fayer, 2008).

PCR - restriction fragment length polymorphism (RFLP) analysis of a number of genes (e.g. small sub unit (SSU) rRNA, *Cryptosporidium* oocysts wall protein (COWP), heat shock protein (HSP) 70) has been used for species identification. Jiang and Xiao (2003) tested the performance of 10 commonly used loci to detect and differentiate *Cryptosporidium* species. The PCR-RFLP of all SSU rRNA gene loci tested amplified and differentiated all species examined (*C. hominis*, *C. parvum*, *C. meleagridis*, *C. felis*, *C. canis*, *C muris* and *C. suis*) but COWP and HSP70 (among others) only amplified and differentiated between *C. hominis*, *C. parvum* and *C. meleagridis* (Smith and Nichols, 2009a). For samples with low numbers of oocysts, PCR amplification of multi-copy genes is preferable as it is a more sensitive method for molecular detection. The SSU rRNA gene loci are therefore suitable targets as there are 20 copies of the same gene present per oocyst (Smith and Nichols, 2009a) and this gene has been targeted for species identification. The Johnson-Nichols nested SSU rRNA assay (Diagnostic assay) (Johnson *et al.*, 1995; Nichols *et al.*, 2003) is useful for analysing samples with low oocysts numbers (Smith, 2008). However, the less sensitive Xiao assay (Xiao *et al.*, 2001a) has the benefit of detecting more species, particularly the 2 most important human pathogens, *C. hominis* and *C. parvum* (Smith, 2008). Therefore, utilising both methods for species identification is extremely powerful.

Although molecular characterisation has allowed for major advances in defining the taxonomy of *Cryptosporidium*, the situation has not been completely elucidated as clarification is still required to determine not only what a species is, but also what are variants of the same species. When significant or consistent sequence differences emerge from the existing genetic data, a new genotype is named after the host from which it was isolated (Xiao *et al.*, 2002). However, a genotype is not a taxon but a partial and temporary descriptor (Fayer, 2008) and as discussed by Fayer (2008), an isolate placed in the genotype category recognises the incomplete knowledge of the isolate while also recognising its uniqueness. It is likely that as research progresses, more genotypes will become validated species. To date, over 40 genotypes have been described, with 7 (*Cryptosporidium* cervine, chipmunk, horse, monkey, pig II, rabbit and skunk genotypes) infecting both immunocompetent and immunocompromised humans (Chalmers *et al.*, 2002; Jirků *et al.*, 2008; Kvac *et al.*, 2009; Smith and Nichols, 2009a).

Understanding the epidemiology of *Cryptosporidium* species and genotypes is important in order to highlight the public health significance of the parasite. In order to

establish the degree of risk associated with *Cryptosporidium*, researchers must identify which species, genotypes and subtypes infect humans, the pathogenicity of each [variations in pathogenicity have been identified not only between species (Okhuysen *et al.*, 1999) but also within species (subtypes) (Cama *et al.*, 2007; Cama *et al.*, 2008)], possible sources of infection (host range) and likely routes of transmission. With extensive information on the epidemiology of each species/genotype, risk factors for infection may be determined allowing for the implementation of control programmes which will disrupt the transmission pathways of the parasite. As there is no validated, effective treatment for cryptosporidiosis, limiting exposure to infectious oocysts appears to be the most appropriate prevention strategy until such treatments are developed.

Genetic variation, along with variation in sources of infection (host range), transmission routes and clinical manifestations, has been identified, not only among species of *Cryptosporidium,* but also within species. Within species variation results in the identification of subtypes. Subtypes have been identified for *C. hominis*, *C. parvum* and *C. meleagridis*, with the majority of studies focusing on the 2 former species. Various tools for the subtyping of these species have been developed, the most common of which is glycoprotein 60 (GP60) sequencing (Strong *et al.*, 2000; Glaberman *et al.*, 2001; Glaberman *et al.*, 2002; Leav *et al.*, 2002a; Peng *et al.*, 2003a). GP60 is a protein on the surface of *Cryptosporidium* sporozoites (Zhu, 2008) and encodes a 60-kilodalton (kDa) precursor glycoprotein (GP) which is cleaved to yield 45kDa (GP45) and 15 kDa (GP15) sporozoite surface glycoproteins (Alves *et al.*, 2003; Akiyoshi *et al.*, 2006), both of which are implicated in parasite adhesion and invasion (Zhu, 2008).

Using GP60, extensive sequence differences have been identified in the non-repeat regions which can categorise *C. hominis* and *C. parvum* into several subtype families (alleles) (Xiao and Ryan, 2008). Six *C. hominis* and 13 *C. parvum* allele families have been identified (Table 1.4, Chapter 1). Each subtype family contains tandem repeats of the serine coding trinucleotide TCA/TCG/TCT at the 5′ end of the gene (Xiao and Ryan, 2008) and most subtype families contain multiple subtypes that differ primarily by the length and type of serine repeats (Akiyoshi *et al.*, 2006). The subtype name usually starts with the species designation (identified by the roman numeral I for *C. hominis* and II for *C. parvum*), subtype family (allele) designation (defined by a lower case letter soon after the species number), followed by the number of TCA (represented by

the letter A), TCG (represented by the letter G) and TCT (represented by the letter T) repeats found in the serine-coding repetitive region (Sulaiman *et al.*, 2005; Cama *et al.*, 2007; Xiao and Ryan, 2008). In addition, subtypes may differ further in the number of a 15 bp repetitive sequence 5′ – AAA/G ACG GTG GTA AGG -3′ (represented by the letter R) shortly downstream of the trinucleotide repeats found in the serine-coding repetitive region (Cama *et al.*, 2007). Thus, the name IbA14G3R1 is a *C. hominis* isolate, of subtype family Ib with 14 TCA repeats, 3 TCG repeats and 1 repeat region. Furthermore, *C. parvum* isolates of subtype family IIc have 5 TCA repeats and 3 TCG repeats for all subtypes and identifiable variations in the 3′ region of the sequences that could be further characterised and therefore IIcA5G3 sequences are arbitrarily designated the letters a, b or c at the end of the subtype name (e.g. IIcA5G3a) (Xiao and Ryan, 2008).

GP60 is the most polymorphic gene identified to date in *C. hominis* and *C. parvum* (Alves *et al.*, 2003; Akiyoshi *et al.*, 2006) and is one of the dominant targets for neutralising antibody responses in humans. Therefore, it is possible to link biological characteristics of the parasite and clinical presentations with the subtype family (Cama *et al.*, 2007; Xiao and Ryan, 2008). Subtyping of *C. hominis* and *C. parvum* has provided a clearer understanding of the transmission dynamics, host specificity and pathogenic potential of these species. Recently, it has been noted that not all human *C. parvum* infections are a result of zoonotic transmission as some *C. parvum* subtypes appear to circulate only in humans (Alves *et al.*, 2003; Mallon *et al.*, 2003). *C. parvum* subtypes IIa and IIc are 2 major GP60 subtype families responsible for most *C. parvum* infections in humans (Xiao and Ryan, 2008). *C. parvum* subtype IIa has been identified in both humans and ruminants while *C. parvum* subtype IIc has been recorded only from humans (Leav *et al.*, 2002a; Alves *et al.*, 2003; Alves *et al.*, 2006; Areeshi *et al.*, 2008; Soba and Logar, 2008; Xiao and Ryan, 2008). Thus, the IIc subtype of *C. parvum* may be exclusive to humans and involved only in anthroponotic transmission.

Genetic heterogeneity has also been observed within *C. meleagridis*. As for *C. hominis* and *C. parvum*, identifying subtypes of *C. meleagridis* may be useful in understanding the epidemiology of the species in various populations and geographical regions. *C. meleagridis* is the third most common infection in humans (McLaughlin *et al.*, 2000; Xiao *et al.*, 2001a; Cama *et al.*, 2003; Tumwine *et al.*, 2003; Akiyoshi *et al.*, 2006; Morse *et al.*, 2007) and is increasingly recognised as an important human pathogen.

Several subtypes of *C. meleagridis* have been described. At the SSU rRNA gene locus, Glaberman (2001) identified 2 subtypes, 1 of which was identical to a subtype isolated from turkeys and sequenced by Xiao (1999a) (genotype 1). Genotype 2 had an additional ATT repeat and a heterogeneous copy of the gene. This heterogeneous copy differed from genotype 1 and the A copy of the gene by single nucleotide mutations at 3 points (Glaberman *et al.*, 2001).

Although now recognised as an important human pathogen, no community-based studies have subtyped *C. meleagridis* isolates. Therefore, further analysis of avian and human isolates from various populations and geographical locations is required in order to fully understand the routes of transmission and public health significance of this species.

Owing to the genetic heterogeneity within *C. hominis*, *C. parvum* and *C. meleagridis*, identification of the isolates at the subtype level is more useful for understanding epidemiology and implementing control measures to decrease risk of infection. As underlined by Smith (2008), the analysis of highly conserved coding regions is required for species analysis, whereas when examining subtypes and identifying sources of infection, risk factors and pathogenicity more discriminatory fingerprinting techniques (such as GP60) are required which can identify subtype families.

The majority of studies which have been conducted in developing countries have focused on the prevalence and temporal variability of *Cryptosporidium* infection but few have determined the species, genotypes and subtypes present. This is particularly true for Nigeria where molecular analysis has never been conducted. Owing to this lack of genotyping data in developing countries, and especially in Nigeria, this chapter aims to identify the species, genotypes and subtypes of *Cryptosporidium* present in a paediatric population in Osun State, Nigeria.

3.2 Materials and Methods

3.2.1 Sample procurement and analysis

Stool samples were collected at 4 time points over a 1 year period from Nigerian children, as described in Section 2.2.3. Positive samples were identified by fluorescent microscopy, following formal-ether concentration and auramine-phenol staining as described in Section 2.2.4. A total of 684 positive samples were identified by microscopy. Due to cost restraints, it was not possible to determine species identity in all 684 positive samples. Therefore, 302 samples were chosen for molecular analysis with 3 criteria utilized for the selection of samples: (1) samples with a high oocyst intensity, (2) positive samples in the month of August as risk factor analysis was carried out at this time and (3) a random selection of positive samples from the 3 remaining time points, resulting in a total of 302 samples.

All molecular preparations and characterisations were carried out in the Scottish Parasite Diagnostic laboratory, NHS Trust, Stobhill hospital, Glasgow, between May and July 2008. Subtyping analysis was carried out in November and December 2008. Therefore, samples collected in September 2006 were stored for almost 34 months prior to molecular analysis. Samples were first purified by a modified water-ether concentration method (Bukhari and Smith, 1995; Nichols *et al.*, 2006b). DNA was released by 15 cycles of freeze-thawing followed by digestion with proteinase K (pK) (Nichols and Smith, 2004; Nichols *et al.*, 2006b). Finally, the samples were subjected to nested PCR and gel electrophoresis to determine whether samples were positive or negative for *Cryptosporidium* oocysts. Samples that were successfully amplified were then digested with specific restriction enzymes to determine the species present (Nichols *et al.*, 2003). These methods are briefly described in the following sections.

3.2.2 Water-ether concentration

Samples were first purified for direct DNA extraction using a modified water-ether concentration method (Bukhari and Smith, 1995; Nichols *et al.*, 2006b). Water-ether treatment allows for the partial purification of oocysts through the removal of debris

and the reduction of PCR inhibitors. For solid stools, a pea-sized amount of the stool sample (200 µl for liquid stools) was transferred to a 1.5 ml microcentrifuge tube containing 100 µl of reverse osmosis (RO) (grade 1) water. The slurry was mixed well using a wooden stick. RO water was added to make up the volume to 700 µl and the tube was vortexed for 30 s. 300 µl of ether was added and the tube was shaken by inversion for 30 s. The tube was then centrifuged in a horizontal fixed angle rotor bench microcentrifuge at 10,000 x g for 1 min. Both the fluid above the fat plug and the fat plug were aspirated to waste using a 1 ml micropipette with a cropped tip (for an enlarged aperture) and the slow suction of a water pump. The tube was vortexed to resuspend the sediment and 1 ml of RO water was added. The sample was washed twice in RO water by spinning at 10,000 x g for 1 min and the supernatant was gently aspirated to waste. Approximately 100 µl remained in the tube after each wash. Following the 2 washes, the tube was vortexed and 1 ml of 1x lysis buffer (LB) (LB; 50 mM Tris HCl pH 8, 1 mM EDTA pH 8, 0.5% SDS) was added and mixed gently. The tube was centrifuged at 10,000 x g for 1 min and the LB wash was repeated. 100 µl remained in the tube following the last wash and the sample was maintained at 4°C prior to DNA extraction.

3.2.3 DNA extraction

DNA was extracted using 15 cycles of freeze-thawing followed by digestion with pK (Nichols and Smith, 2004; Nichols *et al.*, 2006b). 100 µl of oocyst suspension in 1x LB (50mM Tris-HCL, 1ml EDTA and 0.5% SDS, pH 8.0) (remaining following water-ether concentration) was transferred to pre-numbered screw-top, 'O' ring sealed, microcentrifuge tubes. The samples were freeze-thawed 15 times by immersing the tubes alternatively in liquid nitrogen and in a water bath at 65°C for 1 min each. Samples were vortexed every 5 cycles. At the end of the freeze-thaw cycles, the samples were centrifuged at 10,000 x g for 10 s. The supernatant was then transferred to clean 0.5 ml screw-cap tubes. The samples were digested with 200µl/ml proteinase K (i.e. 2 µl of 10 mg/ml pK stock solution to 100 µl of disrupted oocysts) for 3 hrs at 55°C.

Following pK digestion, samples were centrifuged at 14,000 x g for 10 s and the supernatant was treated with a mixture of polyvinylpolypyrrolidone (PVPP) (Sigma, Cat No. P6755) and Chelex 100 (Bio-Rad, Cat No. 142-1253) slurry to minimise PCR

inhibitors. Pre-prepared aliquots of the mixture, each containing 50 µl of 10% PVPP and 50 µl of 10% Chelex suspensions in DNase/RNase free water, were pipetted into 0.5 ml screw-top microcentrifuge tubes and the slurry was allowed to sediment overnight, at 4°C, under gravity. When required, the supernatant of an aliquot of PVPP / Chelex 100 slurry was carefully aspirated with a pipette without disturbing the sedimented slurry and replaced by 50 µl of DNA lysate. The tube was then vortexed, boiled for 10 min, centrifuged (14,000 x g, 1 min) and the supernatant transferred to a clean flip-top tube and frozen at -20°C until used as a template for PCR.

3.2.4 PCR-restriction fragment length polymorphism (RFLP)

3.2.4.1 PCR

Cryptosporidium species were determined by nested PCR-RFLP and/or direct sequencing of the PCR products at 2 SSU rRNA gene loci (Xiao *et al.*, 2001a). Two nested PCR assays were conducted. The first was based on the Nichols-Johnson assay (Diagnostic assay) (Johnson *et al.*, 1995; Ward *et al.*, 2002; Nichols *et al.*, 2003), the second was conducted as described by Xiao (Xiao assay) (Xiao *et al.*, 1999b; Xiao *et al.*, 2000b; Xiao *et al.*, 2001a) (see Appendix 2). A 2-step nested PCR was used as this is more sensitive than the direct PCR assay due to increased amplification at each stage.

C. parvum and *C. hominis* isolates were subtyped by GP60 sequence analysis (Glaberman *et al.*, 2002). When the Glaberman *et al.* (2002) assay failed to produce sufficient amplicon for sequencing, the Sulaiman *et al.* (2005) assay (which produces a 400 bp product compared with the 800 bp product of the Glaberman *et al.* (2002) assay) was used (see Appendix 2 for primer details). In addition, *C. meleagridis* was subtyped by sequence analysis of the SSU rRNA gene as described by Glaberman *et al.* (2001).

PCR amplifications were performed in a GeneAmp® PCR Thermal Cycler, model 9700 (Applied Biosystems, Perkin-Elmer, UK). A laminar flow hood (pre-sterilised with UV light) was used for the set up of all PCR reactions. PCR reaction mixtures, descriptions of the primers, digestion mixtures and thermocycler runs are described in Appendix 2. For the 2-step nested SSU rRNA PCR reactions, total volumes of 50 µl for PCR 1 and

100 µl for PCR 2 were used, with 2 µl of DNA lysate (PCR 1) and first PCR product (PCR 2). The analysis was first tested using 5 µl of DNA lysate and first PCR product. However, non-specific bands were produced on the gel fingerprint and it was determined that 2 µl was the optimum volume for the analysis.

Each PCR run included a positive *Cryptosporidium* DNA control, consisting of *C. parvum* (commercially obtained Iowa isolate from Sterling Parasitology Laboratory, Department of Veterinary Science and Microbiology, University of Arizona) oocyst lysate diluted to 10^3 oocysts/ml and 3 negative controls, consisting of all reagents minus templates. A negative control, consisting of DNase/RNase free water, was prepared in the pre-PCR laboratory, in a hood equipped with UV light. The second and third negative controls, containing lysis buffer, were prepared in the DNA extraction laboratory.

For all PCR assays, 10 µl of secondary PCR products were mixed with 4 µl of bromophenol blue (BPB) and 10 µl of each sample were loaded into the wells of a 1.4% agarose gel. Samples were separated by electrophoresis in 1.4% agarose gels, stained with ethidium bromide, and viewed under UV light, to identify positive PCR reactions.

3.2.4.2 RFLP

The positive secondary PCR products were digested with specific restriction enzymes (Xiao *et al.*, 1999b; Nichols *et al.*, 2003). The positive secondary Diagnostic PCR products were digested simultaneously with *Ase*I and *Dra*I restriction enzymes and the secondary Xiao PCR products were digested separately with *Ase*I and *Ssp*I restriction enzymes (see Appendix 2). Fragments were separated by electrophoresis in a 2% agarose gel, stained with ethidium bromide, and viewed under UV transillumination.

PCR of the SSU rRNA gene locus using Diagnostic primers generate an amplicon of ~435 bp (size varies according to species) (Appendix 2). Endonuclease restriction with *Ase*I and *Dra*I produces the fragments presented in Table 3.1. Similar banding patterns are created for *C. hominis* and *C. parvum* using the Diagnostic assay, and so only the Xiao assay can distinguish between the 2 species. PCR of the SSU rRNA gene locus using Xiao primers generate an amplicon of ~826 bp (the size varies according to

species) (see Appendix 2). Endonuclease restriction with *Ssp*I and *Ase*I produces the fragments presented in Table 3.1.

Table 3.1 Fragments for *Cryptosporidium* species created by the endonuclease restriction enzymes *Ase*I and *Dra*I and *Ase*I and *Ssp*I [modified from Xiao and Ryan (2008)]

***Cryptosporidium* species**	**Diagnostic restriction enzymes**		**Xiao restriction enzymes**	
	***Ase*I**	***Dra*I**	***Ase*I**	***Ssp*I**
C. parvum	219, 104, 112	None	102, 104, 628	11, 12, 111, 254, 449
C. hominis	222, 104, 112	None	70, 104, 102, 561	11, 12, 111, 254, 449
C. meleagridis	47, 171, 104, 112	None	102, 104, 182, 476	11, 108, 254, 449
C. canis	104, 112, 213	None	94, 102, 633	20, 33, 105, 254, 417
Cryptosporidium cervine genotype	47, 104, 112, 171	None	102, 104, 169, 460	11, 371, 453
Cryptosporidium rabbit genotype	222, 104, 112	None	559, 115, 104, 71	473, 267, 109

3.2.5 DNA sequencing and sequence analysis

DNA sequencing was used in some cases to confirm the species/genotype present when RFLP analysis was inconclusive. Sequencing was also used to identify subtypes of *C. hominis*, *C. parvum* and *C. meleagridis*. Amplicons for sequencing of the SSU rRNA (*Cryptosporidium* rabbit genotypes, *C. canis* species, *C. meleagridis* subtypes) and GP60 (*C. hominis* and *C. parvum* subtypes) genes were treated enzymatically with ExoSAP-IT (GE Healthcare) to remove excess dNTPs and primers according to the supplier's instructions. Bi-directional sequencing was performed in an ABI model 3730 sequencer using Big-Dye version 3.1, chemistry and automated capillary DNA sequencer at the Sequencing Service, Dundee University, Scotland (http://www.dnaseq.co.uk/services.html). Bi-directional sequences were aligned using EMBI website tools to obtain a consensus which was manually edited following the sequence chromatogram. The consensus sequence was used to search the GenBank database for similarities using the CBI Blastn tool. ClustalW alignments using the EMBI site was used to compare sequences and the phylogenetic tree was constructed using

the MEGA 4 software. A previously described subtype nomenclature system was used to differentiate subtypes within each subtype family of *C. hominis* and *C. parvum* (Sulaiman *et al.*, 2005).

3.2.6 Optimisation of techniques

3.2.6.1 PVPP/Chelex

Owing to the presence of high concentrations of inhibitors in the stool samples, PVPP and Chelex were used, following DNA extraction, to decrease the concentration of these inhibitors. Eight samples were tested without PVPP and Chelex and the same samples were then tested in the presence of both chemicals. The results showed that without PVPP and Chelex there was non-specific banding and that this was reduced in the presence of PVPP and Chelex, without reducing the strength of the amplicon. Therefore, PVPP and Chelex were incorporated into the DNA extraction method as described in Section 3.2.3. The effectiveness of this method has been reported previously by Smith and Nichols (2009b), when Chelex 100/PVPP treated samples yielded higher concentrations of amplicons for both *Cryptosporidium* DNA and a co-amplified PCR internal control, compared with Chelex 100 or PVPP alone.

3.2.6.2 Oocyst concentration/DNA extraction

A low number (78/302, 25.8%) of samples amplified following PCR and so variations in the techniques described above were tested in an attempt to optimise the methods and thus increase amplification. All samples with an intensity of 2+ and 3+ amplified sufficiently while a much smaller portion of the samples with an intensity of 1+ amplified. It was, therefore, hypothesised that the techniques described above have low sensitivity when analysing samples with low oocyst counts.

Twelve samples which were all positive by AP acid-fast staining and microscopy, and double-checked by a laboratory technician in the Scottish Parasite Diagnostic Laboratory were chosen. Of these, 2 were positive and 10 negative using the molecular techniques described above. These 12 samples were re-processed by immunomagnetic separation (IMS) using Dynal IMS kits (Invitrogen Ltd, Paisley, UK) (Nichols *et al.*, 2006b) instead of water-ether concentration, followed by freeze-

thawing and the PCR techniques described above. Three samples were positive using this approach (i.e. 1 sample previously negative using water-ether concentration was now positive), indicating that IMS may be more sensitive than the water-ether technique. However, only 1 additional sample was amplified and so further optimisation was necessary.

It was hypothesised that the low recovery of oocysts using IMS may have been due to the high pH of the stool samples which may inhibit oocyst recovery from the magnetic beads used in IMS. The pH of each of the 12 stool samples was tested to check for inhibitory pH (Table 3.2). As the pH of the samples was low, even after the IMS buffer was added, glycine was added to the stool sample instead of water when making a slurry for IMS processing. The effects on pH using both glycine and water were compared (Table 3.2). Following IMS, freeze-thawing and the above mentioned PCR techniques, it was shown that there was no difference between both techniques i.e. the addition of glycine (instead of water) increased the pH of the samples (Table 3.2) but did not increase oocyst recovery (Table 3.3).

Bead-beating for the extraction of DNA (McLaughlin *et al.*, 1999) was also tested in order to determine whether this method was more efficient than freeze-thawing. However, when bead beating was used, following water-ether concentration and prior to the aforementioned PCR techniques, it was found that freeze-thawing amplified 1 more sample than bead-beating (Table 3.3).

Table 3.2 Comparison of pH of faecal samples when mixed with water or glycine and IMS buffer

Sample number	pH				
	Water before IMS buffer	Water after IMS buffer	5mls glycine before IMS buffer	10mls glycine before IMS buffer	10mls glycine after IMS buffer
3065	4.92	5.77	5.02	5.10	5.91
1455	5.10	6.16	5.91	5.29	6.55
381	5.06	6.13	5.24	5.35	6.53
728	6.62	6.82	6.57	6.58	7.00
1473	4.70	5.39	4.92	5.07	6.23
787	5.10	5.92	5.23	5.32	6.53
1513	4.64	5.46	4.91	4.99	6.17
2428	4.88	6.15	5.04	5.90	6.14
1544	4.47	4.91	4.73	4.88	5.91
2811	4.56	5.05	5.01	5.13	6.28
547	5.49	6.05	5.77	5.73	6.62
1184	4.41	5.03	4.78	4.81	6.13

Table 3.3 Comparison of methods for concentration and extraction of oocysts for PCR analysis

Sample number	Microscopy	Water ether/ freeze-thaw/ PCR-RFLP	IMS/ freeze-thaw/ PCR-RFLP	IMS (glycine)/ freeze-thaw/ PCR-RFLP	Water ether/ bead beat/ PCR-RFLP
2811	**Positive +**	**Positive +**	**Positive +**	**Positive +**	**Positive +**
1184	**Positive +**	**Positive +**	**Positive +**	**Positive +**	**Positive +**
381	**Positive +**	Negative -	**Positive +**	**Positive +**	Negative -
3065	**Positive +**	Negative -	Negative -	Negative -	Negative -
1455	**Positive +**	Negative -	Negative -	Negative -	Negative -
728	**Positive +**	Negative -	Negative -	Negative -	Negative -
1473	**Positive +**	Negative -	Negative -	Negative -	Negative -
787	**Positive +**	Negative -	Negative -	Negative -	Negative -
1513	**Positive +**	Negative -	Negative -	Negative -	Negative -
2428	**Positive +**	Negative -	Negative -	Negative -	Negative -
1544	**Positive +**	Negative -	Negative -	Negative -	Negative -
541	**Positive +**	Negative -	Negative -	Negative -	Negative -
+ Control	**+ Control**	**+ Control**	**+ Control**	**+ Control**	**+ Control**

3.2.6.3 Discussion of technique optimisation

A low number of samples amplified for species analysis (78/302, 25.8%). It is possible that the long storage time prior to molecular analysis (up to 34 months for some samples) was responsible for low numbers of samples amplifying. However, all samples analysed with an intensity of 2+ and 3+, and few samples with an intensity of 1+ (54/223, 24.2%) amplified, indicating that the molecular techniques employed were not sensitive enough to detect low numbers of oocysts. It was found that by using IMS instead of water-ether concentration, 1 extra sample amplified. However, an increase in amplification of 1 sample was not sufficient to justify the cost of using IMS on all the samples. As discussed by Smith and Nichols (2009b), 'high turbidity, low pH and particulates can reduce the efficiency of IMS and although IMS is more sensitive than the biochemical/biophysical methods used for concentration, particularly at low abundances, it is expensive'.

Extracting DNA by freeze-thawing, for small numbers of oocysts, was preferable to bead-beating. This is consistent with the findings of Smith and Nichols (2009b). These authors indicate that freeze-thawing avoids surface-bound losses and shearing of DNA (which can occur during bead-beating) during extraction. However, the authors mention that DNA extraction by bead-beating can prove advantageous, particularly when oocysts are attached to debris or faecal material.

From the results of the various techniques tested, it seems that the methods used were not sensitive enough to detect low numbers of oocysts. In order to detect oocysts in samples with very low counts, a larger amount of the stool sample needs to be used at the beginning of the process. At present, for the water-ether technique approximately 0.5g of each sample is required. However, if the oocyst count is very low, 0.5g may not contain any oocysts or the count may be so low that the molecular techniques used are not sensitive enough to detect them. Future work in this area needs to focus on a way to increase the amount of faecal material which can be used in the initial stages of the process.

3.3 Results

A total of 302 stool samples were analysed using 2 nested PCR-RFLP assays and / or direct sequencing of PCR products. Of these, 78 (25.8%) samples produced sufficient product for RFLP determination of species. Where required, DNA sequencing of the SSU rRNA gene was used to confirm *Cryptosporidium* species / genotypes. *C. hominis* was detected in 34 samples (43.6%), *C. parvum* in 25 (32.1%), a mixture of *C. parvum* and *C. hominis* in 4 (5.1%), *C. meleagridis* in 5 (6.4%), *Cryptosporidium* rabbit genotype in 5 (6.4%), *Cryptosporidium* cervine genotype in 3 (3.9%) and *C. canis* in 1 (1.3%) (Table 3.4). The identity of 1 isolate could not be determined as the RFLP pattern did not match any known patterns (Plate 3.8). This sample was re-amplified and digested 3 times. On each occasion the same RFLP pattern emerged. However, the isolate could not be sequenced due to the low levels of DNA present. Therefore, the species infecting this child remains unknown. Plates 3.1 – 3.8 present RFLP patterns for each of the species identified. All *Cryptosporidium* rabbit genotypes, and the *C. canis* isolate, were confirmed by sequence analysis of the SSU rRNA gene.

C. hominis was the most common single species infection with 34 (43.6%) isolates identified. *C. parvum* was the second most frequently detected infection occurring in 25 (32.1%) samples. The distribution of *C. hominis* and *C. parvum* species by age, gender and village is presented in Table 3.5. There was no statistically significant association between gender, the village of residence of the child or the age of the child (under 3 yrs vs over 3 yrs) and infection with *C. hominis* or *C. parvum*. Similarly, there was no statistically significant association between infection with *C. hominis* or *C. parvum*, and stool consistency or oocyst intensity (Table 3.5). However, there was a trend for watery stools to be associated with *C. hominis* infections (5 samples) in comparison with *C. parvum* infections (1 sample) (Table 3.5).

Table 3.4 Species and genotypes isolated from Nigerian children

Species / Genotypes	**No. isolated (%)**
C. hominis	34 (43.6)
C. parvum	25 (32.1)
C. hominis/C. parvum	4 (5.1)
C. meleagridis	5 (6.4)
Cryptosporidium rabbit genotype	5 (6.4)
Cryptosporidium cervine genotype	3 (3.9)
C. canis	1 (1.3)
Unknown	1 (1.3)
Total	78 (100)

Table 3.5 Association between gender, age and village of residence with *C. hominis* or *C. parvum* infection

Demographics	Number infected (% prevalence) by *C. hominis*	Number infected (% prevalence) by *C. parvum*	χ^2	p value
Gender				
Male	17 (50.0)	13 (52)	0.001	0.971
Female	17 (50.0)	12 (48)		
Age				
Under 3 yrs	16 (47.1)	13 (52)	0.224	0.621
Over 3 yrs	18 (52.9)	12 (48)		
Village				
Akinlalu	3 (8.8)	6 (24)	5.263	0.153
Ipetumodu	14 (41.2)	13 (52)		
Moro	6 (17.6)	2 (8)		
Edunabon	11 (33.3)	4 (16)		
Stool consistency				
Formed	8 (23.5)	9 (36)	2.410	0.300
Unformed	21 (61.8)	15 (60)		
Watery	5 (14.7)	1 (4)		
Oocyst intensity				
1+	25 (75.8)	20 (80)	0.189	0.910
2+	2 (6.1)	1 (4)		
3+	6 (18.2)	4 (16)		

All species were isolated from both males and females, except for the cervine genotype, which was found only in females. The rabbit genotype was isolated from 5 children, 2 of which were twins (ID 2627 and 2628, Table 3.6). It was not possible to carry out statistical analysis to test for any differences between species other than *C. hominis* and *C. parvum*. Details for children with infections other than *C. hominis* and *C. parvum* are presented in Table 3.6.

Table 3.6 Demographic details, intensity levels of infection and stool consistency for children infected with species other than *C. hominis* and *C. parvum*

Species	ID	Age (Mo)	Gender	Village	Intensity	Stool Consistency
Mixed *C.hominis/C. parvum*	125	34	Female	Ipetumodu	1+	Formed
	696	30	Female	Moro	3+	Unformed
	745	28	Male	Moro	1+	Unformed
	1430	27	Female	Akinlalu	3+	Unformed
C. meleagridis	428	29	Male	Edunabon	1+	Unformed
	734	57	Female	Moro	3+	Unformed
	2614	59	Female	Edunabon	3+	Unformed
	1475	19	Male	Akinlalu	1+	Formed
	494	51	Female	Edunabon	3+	Unformed
Rabbit genotype	58	44	Female	Ipetumodu	1+	Unformed
	2627	37	Male	Edunabon	1+	Unformed
	2628	37	Female	Edunabon	1+	Watery
	2870	52	Male	Akinlalu	1+	Formed
	2907	19.5	Female	Akinlalu	1+	Formed
Cervine genotype	1697	28	Female	Edunabon	3+	Unformed
	165	52	Female	Ipetumodu	3+	Unformed
	421	28	Female	Edunabon	3+	Unformed
C. canis	542	59	Male	Edunabon	3+	Formed
Unknown	3046	40	Female	Edunabon	1+	Unformed

Of the 1636 children that submitted faecal samples for analysis, 349 children submitted samples at each of the 4 time points. Of these 349 children, 153 (43.8%) were never infected with *Cryptosporidium,* 148 (42.4%) were infected on 1 occasion, 43 (12.3%) were infected twice, 5 (1.4%) were infected 3 times and no child was infected on all 4 occasions. Species data were obtained for 2 children who were both infected on 2 occasions. One child was infected with *C. hominis* in May, followed by *C. meleagridis* in August. The second child was infected with *C. parvum* in September and with a mixture of *C. parvum* and *C. hominis* in August. The subtype of *C. parvum* could not be determined by sequence analysis in the mixed sample. Thus, it remains unknown whether or not the subtype of *C. parvum* differed between September and August.

GP60 subtyping was conducted successfully on 28 of 34 *C. hominis* (82%) and 23 of 25 *C. parvum* (82%) isolates. Five subtype families (alleles) of *C. hominis* (Ia, Ib, Id, Ie and 1 novel subtype family) and 4 subtype families of *C. parvum* (IIa, IIc, IIi and 1 unnamed subtype family) were identified (Table 3.7). Subtype families Ia and Ib of *C. hominis* were most commonly detected (10 isolates) and *C. hominis* Ia was also the most genetically diverse consisting of 6 subtypes. Subtype family Ib (10 isolates) and Id (4 isolates) each consisted of 2 genetically distinct subtypes. All 3 isolates of the subtype family Ie consisted of the subtype IeA11G3T3. One isolate (*C. hominis*) was identified which has been previously undescribed. This sequence was deposited in the GenBank database (Accession no. FJ971716), and according to existing nomenclature is ascribed IhA14G1.

Four subtype families (alleles) of *C. parvum* were identified. IIc was the most common (17 isolates) and both IIc and IIa each had 2 subtypes. Both isolates of the subtype family IIi consisted of the subtype IIiA11 and 2 isolates were identified which had 99% similarity to sequences deposited in GenBank with the accession number AY700401. The sequence, (deposited by Hira, K.G., Mackay, M.R., Khan, W.A., Cohen, S., O'Connor, R.M., Leav, B.A., Calderwood, S.B., Ryan, E.T. and Ward, H.D. (2004)) is not published elsewhere, so the isolate has not been ascribed a GP60 subtype (Table 3.7). However, it has been proposed that this subtype family be named IIm (Honorine Ward, pers. comm.).

Phylogenetic analysis was conducted to graphically represent the genetic relationship between the subtype families of *C. hominis* (Figure 3.1 A) and *C. parvum* (Figure 3.1 B). A prototype for each subtype family is included. Tables 3.8 and 3.9 present *C. hominis* and *C. parvum* subtypes isolated from the present study, in addition to information from previous studies which have identified the same subtypes from various geographical locations. This is the first study to isolate a number of *C. hominis* subtypes which have not been previously deposited in GenBank (Tables 3.8 and 3.9).

In addition, 3 isolates of *C. meleagridis* were subtyped by sequence analysis of the SSU rRNA gene fragment. All 3 isolates belonged to the subtype, Type 1, as described by Glaberman *et al.* (2001) and Xiao *et al.* (1999a) (Table 3.7).

Table 3.7 Subtypes of *C. hominis*, *C. parvum* and *C. meleagridis* identified from children in rural Nigeria

Species, subtype family, subtype	**No. of children infected**
C. hominis	34 (28 subtyped)
Ia	10
IaA18R2	3
IaA22R2	1
IaA24R2	2
IaA25R2	2
IaA28R2	1
IaA21R1	1
Ib	10
IbA10G2	3
IbA13G3	7
Id	4
IdA11	2
IdA17	2
Ie	3
IeA11G3T3	3
Ih (Novel subtype)	1
IhA14G1	1
C. parvum	25 (23 subtyped)
IIa	2
IIaA15G2R1	1
IIaA16G1R1	1
IIc	17
IIcA5G3a	9
IIcA5G3b	8

Table 3.7 Contd

Species, subtype family, subtype	No. of children infected
IIi	2
IIiA11	2
IIm	2
IImA10G1	2
C. meleagridis	5 (3 subtyped)
Type 1	3

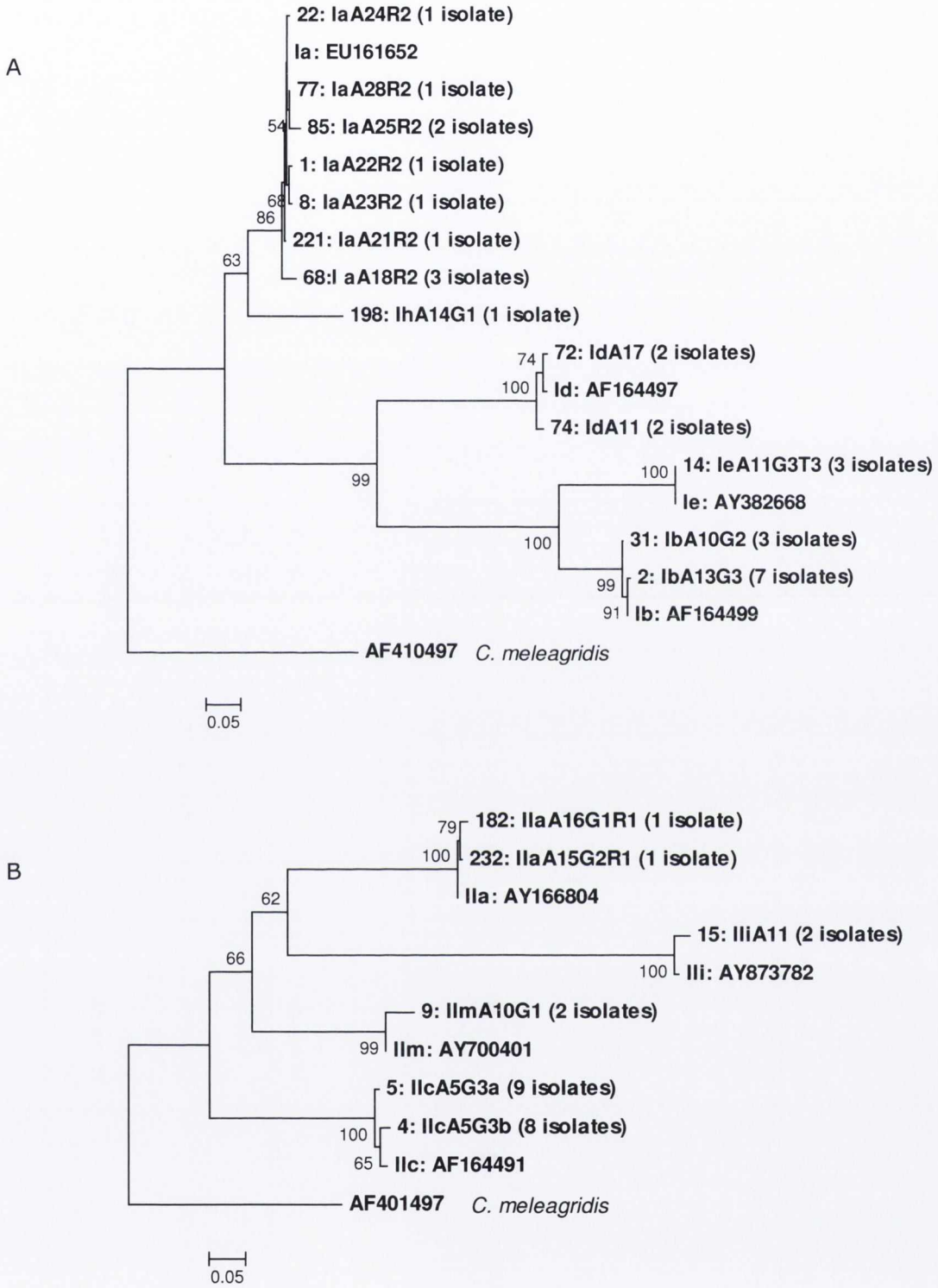

Figure 3.1 Phylogenetic analysis of *C. hominis* (A) and *C. parvum* (B) subtypes from Nigerian children and sequences (with accession numbers) previously deposited in GenBank using neighbour-joining analysis of the GP60 gene. The total number of isolates sequenced for each subtype allele family is included in brackets. Values at the end of each branch represent the sample number of the isolate. Values on branches are percentage bootstraps values using 1000 replicates. Bootstrap values greater than 50% are shown.

Table 3.8 Host, location and accession number for *C. hominis* subtypes which were found in these Nigerian children and also previously deposited in GenBank

Subtype (No. isolated in the current study)	No. isolates deposited previously in GenBank	Host	Location	Accession No.	Reference
IaA18R2 (3)	0	N/A	N/A	N/A	N/A
IaA21R1 (1)	0	N/A	N/A	N/A	N/A
IaA24R2 (2)	0	N/A	N/A	N/A	N/A
IaA25R2 (2)	0	N/A	N/A	N/A	N/A
IaA28R2 (1)	0	N/A	N/A	N/A	N/A
IaA22R2 (1)	1	Humans	UK	EU161652	Chalmers *et al.* (2008)
IbA10G2 (3)	31	Humans	Australia, Slovenia, Jamaica, Netherlands, UK, Kuwait	FJ861209 - FJ861216, FJ861220 - FJ861238 AM988862, EU141720, EF576982, EU161654, AY738187,	Sulaiman *et al.* (2005); Chalmers *et al.* (2008); Gatei *et al.* (2008); Soba and Logar, (2008); Wielinga *et al.* (2008); Waldron *et al.* (2009)
IbA13G3 (7)	1	Humans	Peru	EF035554	Cama *et al.* (2006)
IdA11 (2)	0	N/A	N/A	N/A	N/A
IdA17 (2)	2	Humans	Kenya, Netherlands	EU146137, EF576980	Gatei *et al.* (Unpublised); Wielinga *et al.* (2008)
IeA11G3T3 (3)	3	Humans	Australia, India, Kuwait	FJ839874, DQ665689, Y738184	Gatei *et al.* (Unpublised); Sulaiman *et al.* (2005); Waldron *et al.* (2009)

Table 3.9 Host, location and accession number for *C. parvum* subtypes which were found in these Nigerian children and also previously deposited in Genbank

Subtype (No. isolated in the current study)	No. isolates deposited in GeneBank	Host	Location	Accession No.	References
IIaA15G2R1 (1)	11	Humans	Australia, Slovenia, Netherlands, US, Kuwait	FJ861283, AM988865, AM937015, AM937016, EF576979, DQ640630, AY738190	Sulaiman *et al.* (2005); Feltus *et al.* (2006); Soba and Logar (2008); Wielinga *et al.* (2008); Waldron *et al.* (2009)
		Cattle/ calves	UK, Canada, US, N. Ireland, Netherlands, Brazil	EU200445, DQ192503, DQ630578, DQ648546, EF576968, EF175937	Feltus *et al.* (2006); Trotz-Williams *et al.* (2006); Meireles *et al.* (2007); Thompson *et al.* (2007); Xiao *et al.* (2007a); Wielinga *et al.* (2008); Brook *et al.* (2009)
		Sheep/ Lambs	Netherlands, Spain	EF576974, EU549719	Quilez *et al.* (2008); Wielinga *et al.* (2008)
		Capybara	Brazil	EF175939	Meireles *et al.* (2007)
IIaA16G1R1 (1)	3	Cattle	Netherlands, Canada	EF576969, DQ192504	Trotz-Williams *et al.* (2006); Wielinga *et al.* (2008)
		Humans	US	DQ640633	Feltus *et al.* (2006)

Table 3.9 Contd

Subtype (No. isolated in the current study)	No. isolates deposited in GeneBank	Host	Location	Accession No.	References
IIcA5G3a (9)	1	Humans	Kuwait	AY738195	Sulaiman *et al.* (2005)
IIcA5G3b (8)	2	Humans	Peru	EU095266	Cama *et al.* (2008)
IIcA5G3	4	Humans	Australia, Slovenia, Jamaica	FJ839876, EF025581, AM947935, EU141721	O'Brien *et al.* (2008); Soba and Logar (2008); Waldron *et al.* (2009)
IIiA11 (2)	2	Humans	Uganda	AY873780	Akiyoshi *et al.* (2006)
IImA10G1 (2)	1	Humans	Bangladesh	AY700401	Hira *et al.* Unpublished

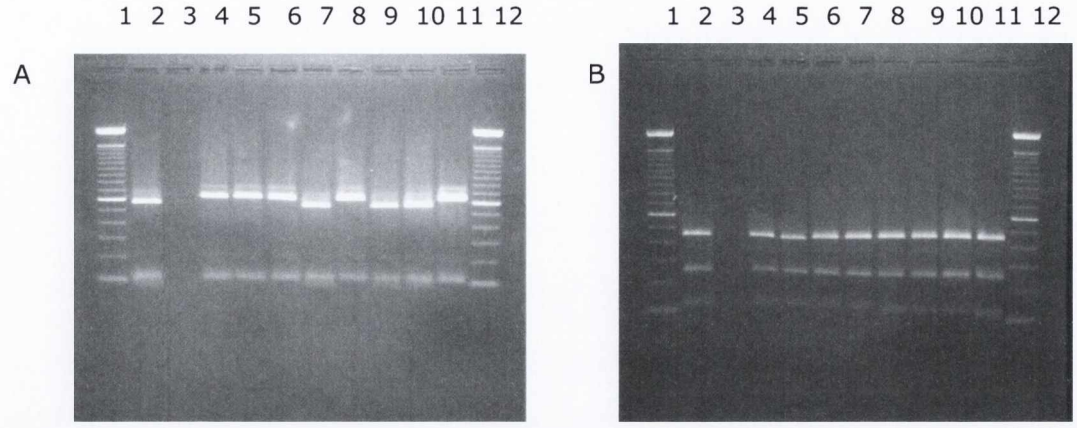

Plate 3.1 RFLP digest using Xiao amplicon and *Ase*I (A) and *Ssp*I (B) restriction enzymes. Lanes 1, 12: DNA ladder, lane 2: *C. hominis*, lane 3: No amplification, lanes 4/6: *C. parvum*, lane 7: *C. hominis*, lane 8: *C. parvum*, lanes 9, 10: *C. hominis*, lane 11: Positive control

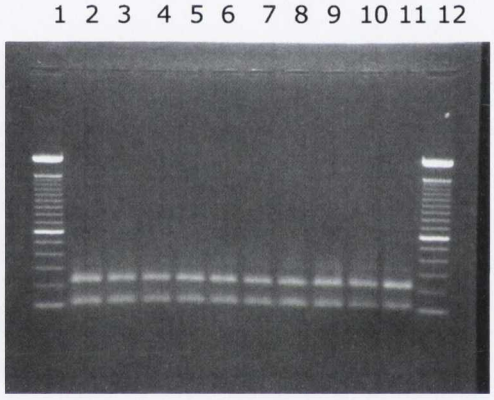

Plate 3.2 RFLP digest using Diagnostic amplicon and *Ase*I and *Dra*I restriction enzymes. Lanes 1, 12: DNA ladder, lanes 2/10: *C. hominis* OR *C. parvum*, lane 11: Positive control

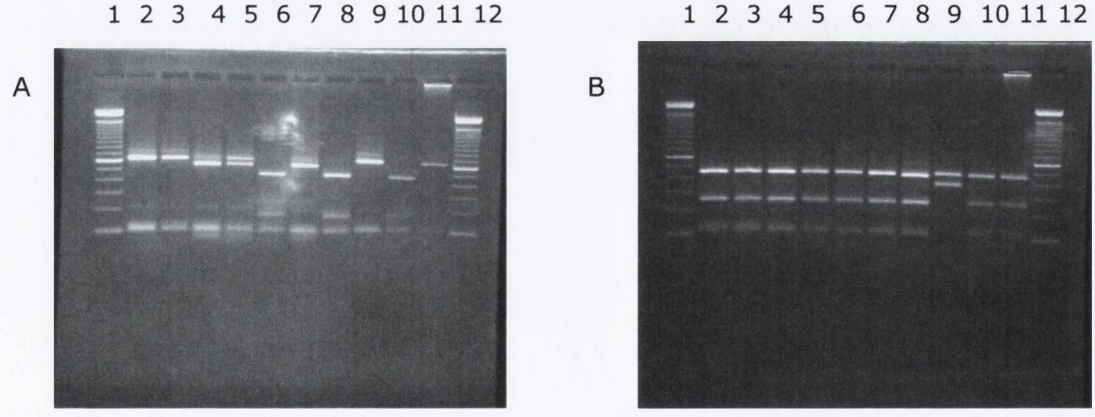

Plate 3.3 RFLP digest using Xiao amplicon and *Ase*I (A) and *Ssp*I (B) restriction enzymes. Lanes 1, 12: DNA ladder, lanes 2, 3: *C. parvum*, lanes 4, 5: *C. parvum* AND *C. hominis*, lane 6: *C. meleagridis*, lane 7: *C. parvum* AND *C. hominis*, lane 8: *C. meleagridis*, lane 9: *Cryptosporidium* cervine genotype (possibly a mixture here but confirmed *C. canis* by sequence analysis of SSU rRNA gene), lane 10: *C. meleagridis*, lane 11: Positive control

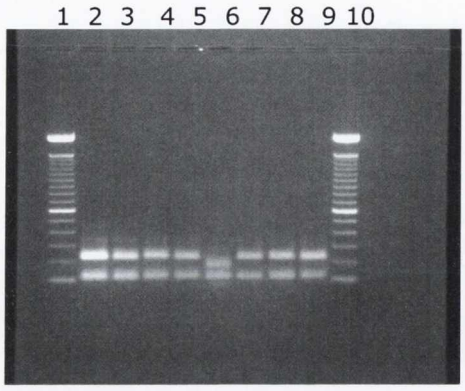

Plate 3.4 RFLP digest using Diagnostic amplicon and *Ase*I and *Dra*I restriction enzymes. Lane 1, 10: DNA ladder, lanes 2/5: *C. hominis* OR *C. parvum*, lane 6: C. meleagridis, lanes 7, 8: *C. hominis* or *C. parvum*, lane 9: Positive control

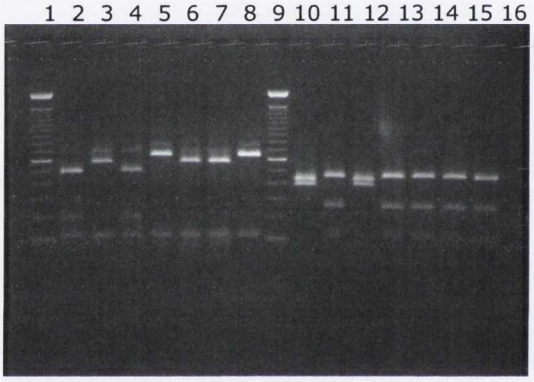

Plate 3.5 RFLP digest using Xiao amplicon and *Ase*I and *Ssp*I restriction enzymes. Lanes 2/8: *Ase*I primers, lanes 10/16 *Ssp*I primers. Lanes 1, 9: DNA ladder, lanes 2, 10: *Cryptosporidium* cervine genotype, lanes 3, 11: *C. hominis*, lanes 4, 12: *Cryptosporidium* cervine genotype, lanes 5, 13: *C. parvum*, lanes 6, 14: *C. hominis*, lanes 7, 15: *C. hominis*, lanes 8, 16: Positive control

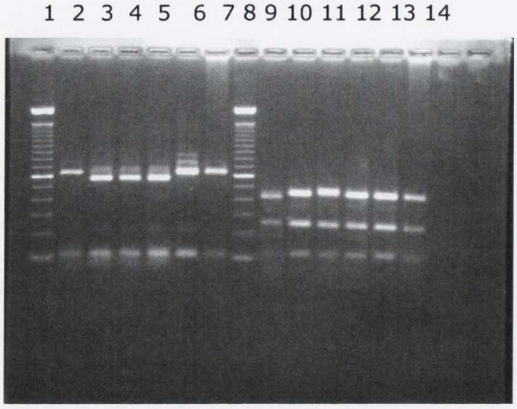

Plate 3.6 RFLP digest using Xiao amplicon and *Ase*I and *Ssp*I restriction enzymes. Lanes 2-7 *Ase*I primers, lanes 9-14 *Ssp*I primers. Lanes 1, 8: DNA ladder, lanes 2, 9: *C. canis* (confirmed by sequence analysis of SSU rRNA gene), lanes 3, 10: *C. hominis*, lanes 4, 11: *Cryptosporidium* rabbit genotype (confirmed by sequence analysis of SSU rRNA gene), lanes 5, 12: *C. hominis*, lanes 6, 13: *C. parvum*, lanes 7, 14: Positive control

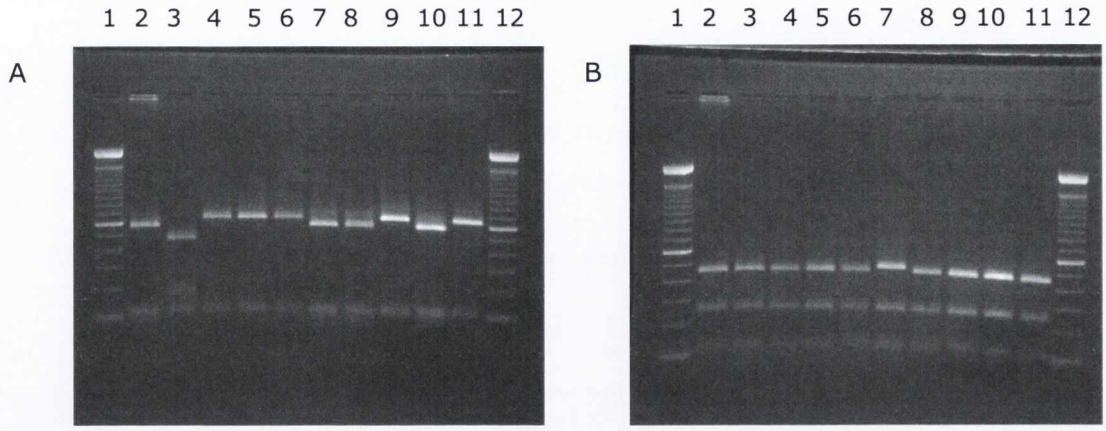

Plate 3.7 RFLP digest using Xiao amplicon and *Ase*I (A) and *Ssp*I (B) restriction enzymes. Lanes 1, 12: DNA ladder, lane 2: *C. hominis*, lane 3: *C. meleagridis*, lanes 4/6: *C. parvum*, lane 7: *Cryptosporidium* rabbit genotype (confirmed by sequence analysis of SSU rRNA gene), lane 8: *C. hominis*, lane 9: *C. parvum*, 10: *C. hominis*, lane 11: Positive control

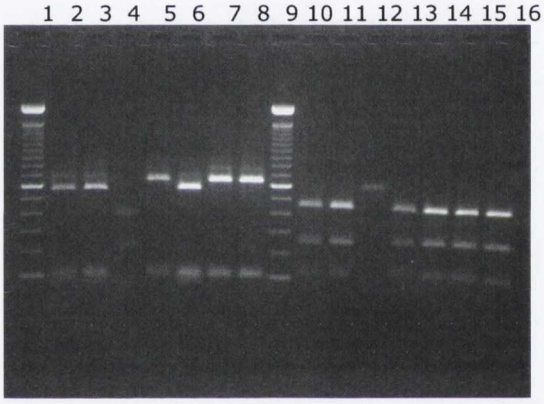

Plate 3.8 RFLP digest using Xiao amplicon and *Ase*I and *Ssp*I restriction enzymes. Lanes 2/8: *Ase*I primers, lanes 10/16: *Ssp*I primers. Lanes 1, 9: DNA ladder, lanes 2, 10, 3, 11: *C. hominis*, lanes 4, 12: **Unknown**, lanes 5, 13: *C. parvum*, lanes 6, 14: *C. hominis*, lanes 7, 15: *C. parvum*, lanes 8, 16: Positive control

3.4 Discussion

This is the first study to genetically characterise *Cryptosporidium* species in Nigeria. Our results indicate that children were infected with a diverse range of species, genotypes and subtypes, including a novel subtype of *C. hominis*. Previous research carried out in Nigeria has focused on the prevalence of infection in various populations (Reinthaler *et al.*, 1987; Kwaga *et al.*, 1988; Oyerinde *et al.*, 1989; Okafor and Okunji, 1994; Okafor and Okunji, 1996; Nwabuisi, 2001; Nwokediuko *et al.*, 2002; Banwat *et al.*, 2003; Okodua *et al.*, 2003) but none have identified the species present.

3.4.1 Species and genotypes

The current study isolated 6 species/genotypes, namely, *C. hominis*, *C. parvum*, mixtures of *C. hominis* and *C. parvum*, *C. meleagridis*, *C. canis*, the rabbit genotype and the cervine genotype. In Africa, genetic characterisation of *Cryptosporidium* species is generally lacking. Species data have been collected from just 5 countries; Kenya, Malawi, Uganda, Equatorial Guinea and S. Africa and include *C. hominis*, *C. parvum*, *C. canis*, *C. felis*, *C. meleagridis* and *C. muris* (Morgan *et al.*, 2000a; Leav *et al.*, 2002a; Gatei *et al.*, 2006a; Morse *et al.*, 2007; Blanco *et al.*, 2009). However, only in Kenya have all 6 species been reported (Gatei *et al.*, 2006a) [A putative case of *C. andersoni* was also identified in Malawi (Morse *et al.*, 2007)]. The isolation of 6 *Cryptosporidium* species / genotypes in this paediatric population in Nigeria highlights the heterogeneity of *Cryptosporidium* in these children. This is the first time that both the rabbit and the cervine genotypes have been isolated in Africa.

C. hominis was the dominant species identified in our study. Dominance of *C. hominis* is consistent with studies from other developing countries such as Peru (Xiao *et al.*, 2001a), Kenya (Gatei *et al.*, 2006a), India (Ajjampur *et al.*, 2007), Malawi (Peng *et al.*, 2003b; Morse *et al.*, 2007) and Uganda (Tumwine *et al.*, 2003; Akiyoshi *et al.*, 2006) indicating that anthroponotic transmission may play a major role in the epidemiology of *Cryptosporidium* in these areas. A relatively high level of *C. parvum* was also found in the current study and this finding is in contrast to studies from other developing regions where *C. parvum* infections are generally much lower than that of *C. hominis* (Xiao *et al.*, 2001a; Cama *et al.*,

2003; Peng *et al.*, 2003b; Tumwine *et al.*, 2003; Tumwine *et al.*, 2005; Akiyoshi *et al.*, 2006; Das *et al.*, 2006; Ajjampur *et al.*, 2007; Cama *et al.*, 2007; Cama *et al.*, 2008). Higher levels of *C. parvum* are consistent with studies from Kuwait, Equatorial Guinea and also developed countries such as France, Portugal and the UK (McLaughlin *et al.*, 2000; Guyot *et al.*, 2001; Alves *et al.*, 2003; Sulaiman *et al.*, 2005). Variations in the distribution of *Cryptosporidium* species in humans are considered an indication of differences in infection sources (Sulaiman *et al.*, 2005). The dominance of *C. parvum* in developed countries may reflect differences in farming techniques, with developed nations adopting intensive (rather than extensive) agricultural practices. Keeping large numbers of cattle on small areas of land is likely to be conducive to the spread of infections such as cryptosporidiosis and this zoonotic transmission of *C. parvum* was highlighted in the UK when strict restrictions on countryside access and movement of livestock were imposed in 2001 due to the outbreak of Foot and Mouth disease. These restrictions resulted in reduced numbers of humans coming in contact with livestock and a dramatic decrease (up to 63%) in the incidence of *C. parvum* occurred as a result (Hunter *et al.*, 2003; Smerdon *et al.*, 2003). Extensive farming practiced in most developing countries may reduce the risk of zoonotic infection from livestock. Similarly, higher levels of *C. hominis* infection in developing regions is likely owing to lower hygiene and sanitation standards in comparison to developed countries, leading to increased anthroponotic transmission.

However, while it is plausible that the presence of *C. hominis* in a population indicates exclusively anthroponotic sources of infection (owing to the host specificity of *C. hominis*), the same cannot be said of *C. parvum* and zoonotic sources of infection. Subtype analysis has now indicated that the *C. parvum* subtype IIc is anthroponotic, circulating only in humans, thus, studies which lack subtyping data cannot conclude that high levels of *C. parvum* indicate a zoonotic source of infection. In addition, in Kuwait, the most common subtypes identified were *C. parvum* IIa and IId with few IIc subtypes isolated (Sulaiman *et al.*, 2005). *C. parvum* IIa has been isolated from both humans and cattle while IId has been isolated from AIDS patients and cattle in Portugal (Alves *et al.*, 2001). Therefore, even with the aid of subtyping data, it is not always clear whether anthroponotic or zoonotic sources are responsible for infection. It is therefore essential that molecular data be accompanied with environmental sampling and risk factor analysis in order to fully elucidate the epidemiology of *Cryptosporidium* infection.

In the present study, *C. hominis* and *C. parvum* were found in similar numbers in both males and females, and between children under and over the age of 3 years. This is analogous to the findings reported from in southern India, which found no significant differences in age and gender between *C. hominis*-infected children and those infected with other species (Ajjampur *et al.*, 2007).

In addition to the isolation of *C. parvum* and *C. hominis*, *C. meleagridis*, *C. canis*, *Cryptosporidium* cervine genotype and *Cryptosporidium* rabbit genotype were identified in the current study. *C. meleagridis* was first identified in turkeys in 1955 (Slavin, 1955) and since then has been reported as a cause of cryptosporidiosis in >30 species of birds (Glaberman *et al.*, 2001). Once believed to be limited to avian hosts, *C. meleagridis* is now recognised as the third most common *Cryptosporidium* infection in both immunocompetant and immunocompromised humans (Pedraza-Diaz *et al.*, 2000; Guyot *et al.*, 2001; Gatei *et al.*, 2003) (although much less common than *C. parvum* and *C. hominis*) with prevalence rates in developing countries (e.g. Kenya, Uganda, Equatorial Guinea, Malawi and Peru) ranging from 2.9 to 16.7% (Morgan *et al.*, 2000a; Xiao *et al.*, 2001a; Cama *et al.*, 2003; Gatei *et al.*, 2003; Tumwine *et al.*, 2003; Tumwine *et al.*, 2005; Akiyoshi *et al.*, 2006; Cama *et al.*, 2007; Morse *et al.*, 2007; Cama *et al.*, 2008; Blanco *et al.*, 2009). Similarly, *C. meleagridis* was the third most common infection found in the present study, infecting 5 (6.3%) children.

C. canis is primarily associated with infections in dogs and foxes but has been isolated from patients in the USA, Thailand, Kenya, Peru, New Zealand and France (Xiao *et al.*, 2001a; Cama *et al.*, 2003; Xiao *et al.*, 2004; Gatei *et al.*, 2006a; Xiao *et al.*, 2007b; Cama *et al.*, 2008). Although possible associations between human cryptosporidiosis and contact with dogs have been identified (Xiao *et al.*, 2007b) other studies have found no association (Goh *et al.*, 2004) and have even found contact to be protective (Robertson *et al.*, 2002). Therefore, both zoonotic and anthroponotic transmission pathways of *C. canis* are plausible. *C. canis* has been found on only 1 occasion previously in Africa, when it was isolated from 3 children in Kenya (Gatei *et al.*, 2006a). In the present study 1 isolate of *C. canis* was identified.

The cervine genotype was first identified in storm waters in New York state and has since been associated with infections in domestic and wild ruminants, rodents and primates (Xiao *et al.*, 2004). The cervine genotype was first reported in humans from Canada (Ong *et al.*, 2002) and has since been recorded sporadically

from individuals in Ontario, the USA, Slovenia and England (Xiao and Ryan, 2008). According to Xiao and Ryan (2008), the increasing number of humans infected may be related to the wide host range of the cervine genotype. The present study is the first in Africa to isolate the cervine genotype.

Finally, the rabbit genotype, which was first identified in rabbits in China (Xiao *et al.*, 2002) and was subsequently isolated from rabbits from the Czech Republic (Ryan *et al.*, 2003), was first isolated in humans in 2008 in an immunocompetent woman in England and has since been responsible for a waterborne outbreak in the UK (Chalmers *et al.*, 2009b). No other cases of infection with the rabbit genotype have since been identified in humans. The rabbit genotype is similar but not identical to *C. hominis*, with RFLP patterns at the COWP locus, sharing 99.2% similarity at the SSU rRNA locus and 99.7% at the HSP locus (Ryan *et al.*, 2003; Robinson *et al.*, 2008b), therefore it may not be surprising that human infections have been identified. Two of the 5 children infected with the rabbit genotype were twins, indicating that oocysts may have been transmitted anthroponotically between the siblings. Alternatively, it is possible that the children contracted the infection from the same source.

The present study also observed the presence of mixed *C. hominis* and *C. parvum* infections within the same child. Mixed infections have previously been isolated from children in tropical countries such as Uganda, Malawi, Kuwait, India and Peru (Tumwine *et al.*, 2003; Sulaiman *et al.*, 2005; Tumwine *et al.*, 2005; Akiyoshi *et al.*, 2006; Gatei *et al.*, 2007; Morse *et al.*, 2007; Cama *et al.*, 2008) with the majority of studies isolating mixed infections of *C. hominis* and *C. parvum*. However, in India, 2 children were infected with a mixture of *C. hominis* and *C. meleagridis*, while in Peru 1 child was found to be infected with both *C. canis* and *C. meleagridis* (Gatei *et al.*, 2007; Cama *et al.*, 2008). In the UK, 2414 faecal samples were studied and mixtures of *C. hominis* and *C. parvum* represented nearly 1% of all cases (Nichols, 2008).

Nonetheless, the occurrence of mixed infections may well be underappreciated as they are often difficult to identify using molecular methods. Often the dominant species in the sample will be amplified by PCR and the second species may remain undiscovered. For example, 1 study demonstrated that the SSU rRNA PCR-RFLP tool had only a 31 to 74% success rate in detecting mixtures of *C. hominis* and *C. parvum* oocysts (Smith, 2008). Mixed infections are thought to represent exposure to sources of contamination that contains multiple pathogens such as sewage (Nichols, 2008). As discussed by Smith (2008), the inability to detect

mixed infections can compromise our understanding of the epidemiology of *Cryptosporidium* especially since it is now recognised that different species are linked to differences in clinical manifestations and oocyst excretion dynamics.

In addition, our study indicates that an individual child can be infected with different species at different times of the year. One child was infected with *C. hominis* in May followed by *C. meleagridis* in August, while a second child was infected with *C. parvum* in September and with a mixture of *C. parvum* and *C. hominis* in August. Repeat infections with both homologous and heterologous species have been previously identified in Peru (Xiao *et al.*, 2001a; Cama *et al.*, 2008). Similarly, volunteers experimentally infected with *C. parvum* bovine genotypes were susceptible to challenge 1 year later, although subsequent exposure was not necessarily associated with symptoms (Okhuysen *et al.*, 1999). As discussed by Xiao *et al.* (2001a), acquired immunity against *Cryptosporidium* may only be partial or short lived allowing repeat infections to occur.

3.4.2 Subtypes

Of the 6 *C. hominis* subtype families described, 4 were isolated in the present study (Ia, Ib, Id and Ie), belonging to 12 subtype allele families. These common subtype families are found in humans worldwide, however, the subtypes IaA18R2, IaA21R2 IaA24R2 IaA25R2, IaA28R2 and IdA11 have not previously been deposited in GenBank. This may indicate the occurrence of new subtypes in the *C. hominis* families Ia and Id. Alternatively, identical subtypes may not have been identified in GenBank because not all isolates deposited are assigned a subtype name. Either the authors do not include a description of the subtype or the subtype may have been deposited before the existing nomenclature was defined.

The remaining *C. hominis* subtypes have been previously described in other studies. The subtype IaA22R2 was isolated on only 1 occasion previously from a human in the UK while IbA10G2 has been isolated on many occasions in humans from Australia, Slovenia, Jamaica, the Netherlands, the UK and Kuwait (Table 3.8). The subtype IbA13G3 was isolated on 1 occasion previously in a HIV-infected individual in Peru and the subtype IdA17 was isolated on 2 occasions in children in Kenya, while IeA11G3T3 was isolated from humans in Australia, India and Kuwait.

In addition, 1 novel subtype of *C. hominis* was isolated in the current study and was described fully as subtype IhA14G1, in accordance with previously described nomenclature (Sulaiman *et al.*, 2005).

Of the 13 *C. parvum* subtypes described to date, we report the presence of subtypes IIa, IIc, IIe and IIi in addition to an unnamed subtype family, belonging to 6 subtype allele families. The sequence for the unnamed subtype has been deposited in GenBank but the work is unpublished (Hira *et al.*, Unpublished). It has been proposed that this subtype be named IIm (Honorine Ward, pers. comm.).

Subtype family IIa has been isolated from both ruminants and humans and coupled with IIc is the most common *C. parvum* subtype found to infect humans worldwide (Xiao and Ryan, 2008). IIaA15G2R1 has been isolated on 11 occasions previously from humans in Australia, Slovenia, the Netherlands, the US and Kuwait, from cattle and calves in the UK, Canada, the US, N. Ireland, the Netherlands and Brazil, sheep and lambs from the Netherlands and Spain and from capybara in Brazil. The subtype IIaA16G1R1 was isolated less frequently on just 3 occasions in cattle from the Netherlands and Canada and humans from the US, while IIcA5G3a was isolated in children from Kuwait and IIcA5G3b from children in Peru. As authors do not always put the letters a, b, c and d at the end of this subtype name a search was also conducted in GenBank for IIcA5G3. This subtype was previously identified in humans in Australia, Slovenia and Jamaica (Table 3.9). The subtype family IIi is rare, found on only 1 occasion previously in children in Uganda (Akiyoshi *et al.*, 2006). Likewise, IIm was identified on only 1 occasion, with the exception of the current study, in Bangladeshi children (Hira *et al.*, Unpublished) (Table 3.9).

Prior to the development of subtyping techniques, the presence of high levels of *C. parvum* would have suggested that zoonotic transmission was as important as anthroponotic transmission in this region in Nigeria. However, results from the current subtyping analysis indicate that subtype IIc is the dominant *C. parvum* subtype in this population, accounting for 17 of 25 (68%) *C. parvum* subtypes identified. As IIc is primarily limited to human infections, this would indicate that the primary source of infection in these Nigerian children is anthroponotic, although this was not determined epidemiologically.

Subtyping data are deficient in African countries with information available from just 3 studies which were conducted in South Africa, Malawi and Uganda (Leav *et*

al., 2002a; Peng *et al.*, 2003b; Akiyoshi *et al.*, 2006). In these studies, *C. hominis* subtype families included Ia, Ib, Id and Ie while the *C. parvum* subtype families isolated were IIc, IIe, IIg, IIh and IIi. This indicates that the present study is the first to isolate the *C. parvum* IIa and IIm subtype families from humans in Africa.

Subtyping of 3 *C. meleagridis* isolates in the current study indicated the presence of *C. meleagridis* Type 1 using SSU rRNA gene sequencing. Very few studies have subtyped *C. meleagridis.* The Type 1 genotype has previously been isolated from turkeys and humans (Xiao *et al.*, 1999a; Glaberman *et al.*, 2001). A second genotype has been observed at the SSU rRNA gene, however this subtype was not isolated in our study. Further collaborative studies are required to address the epidemiological significance of *C. meleagridis* subtyping. Molecular epidemiological analysis of *C. meleagridis* requires the identification and genotyping of avian, human and environmental isolates in order to understand the significance of the presence of subtypes. These data in combination with patient information may establish whether a specific case of human infection is attributable to a given animal reservoir directly or through the contamination of water or food by animal faces or indeed in involved in anthoponotic transmission (Glaberman *et al.*, 2001).

3.4.3 Public health significance of isolated species, genotypes and subtypes

As previously discussed, different species, genotypes and even subtypes will have varying host ranges (and thus differing sources of infection and transmission routes). Therefore, the public health significance may vary depending on the species, genotypes and subtypes present. In addition, recent studies have indicated that clinical symptoms induced by *Cryptosporidium* in the host vary, not only among *Cryptosporidium* species, but also among subtypes of the same species (Bushen *et al.*, 2007; Cama *et al.*, 2007; Cama *et al.*, 2008). Thus, the high diversity of subtypes isolated from these children in Nigeria may result in differences in clinical manifestations and may also indicate the presence of various sources of infection for this population.

Differences in clinical manifestations among the species, genotypes and subtypes could not be determined conclusively in our study due to a lack of clinical data and low sample sizes of the less common species and subtypes. However, stool consistency and oocyst intensity was noted. Stool consistency may be useful as an

indication of diarrhoeal status, as formed stools can indicate that diarrhoea is absent, while watery stools indicate the presence of diarrhoea. However, unformed stools represent ambiguity over whether or not a child was suffering from diarrhoea as these stools may be a result of diarrhoea or may simply depend on the diet of the child. In addition, stool samples in this study were not tested for the presence of all enteric parasites and so even if a child is suffering from diarrhoea, we cannot rule out the chance that the watery stool was caused by another infection. Thus, stool consistency alone cannot give precise information on the pathology of *Cryptosporidium* species but may be helpful in conjunction with oocyst intensity data and information from previous studies in order to give an indication of differences in pathology among the species.

In the current study, there was no statistically significant association between stool consistency and infection, nor between stool consistency and intensity of infection (see Chapter 2, Section 2.3), indicating that infection is not associated with watery stools, and that intensity of infection does not alter the stool consistency. In addition, neither stool consistency nor oocyst intensity was associated with either *C. hominis* or *C. parvum* infections. This indicates that asymptomatic infection was common in this population and that there is little difference in the clinical outcomes between *C. hominis* and *C. parvum* infections. However, very few watery stools were collected and so lack of statistical significance may be simply a consequence of low sample size.

From previous studies, *C. hominis* Ib was implicated as the most pathogenic of the *C. hominis* isolates as it was associated with diarrhoea, nausea, vomiting and general malaise, while other *C. hominis* subtypes were associated only with diarrhoea (Cama *et al.*, 2008). *C. hominis*, as a species, is considered more pathogenic than *C. parvum* as studies have shown that *C. hominis* infection leads to increased oocyst shedding, intensity and duration (McLaughlin *et al.*, 1999; Xiao *et al.*, 2001a; Bushen *et al.*, 2007; Cama *et al.*, 2008). In addition, in Brazilian children, *C. hominis* infections resulted in a significantly greater impact on linear growth in comparison to *C. parvum* infections. *C. hominis* infection was not associated with watery stools nor with increased oocyst intensity in the current study. Previous surveys, however have indicated that even in children without diarrhoea, infection can lead to measurable growth shortfalls especially in childhood infections (Checkley *et al.*, 1997; Checkley *et al.*, 1998).

Few studies have focused on the pathogenicity of *C. meleagridis* for humans. In Peru, *C. meleagridis* was isolated in HIV-positive adults but was not associated

with clinical manifestations (Cama *et al.*, 2007). In contrast, *C. meleagridis* infections in Peruvian children were associated with diarrhoea (although not as severe as *C. hominis*) (Cama *et al.*, 2008). Similarly, a Portuguese study found that *C. meleagridis*-infected patients had diarrhoea despite being treated with HAART and although *C. meleagridis* was associated with low oocyst loads, 2 of the 3 patients infected with *C. meleagridis* died (Matos *et al.*, 2004). Thus, *C. meleagridis* must be considered pathogenic to humans, especially those with a compromised immune system. Inconsistencies among studies relating to the pathogenic potential of *C. meleagridis* may be owing to variations in the pathogenicity of subtypes, however further subtyping work on large number of samples is required before the public health significance of subtypes of *C. meleagridis* can be determined.

Similarly, very little research has been conducted evaluating the pathogenic potential of *C. canis* for humans. In HIV-positive individuals in Peru, *C. canis* (pooled with *C. felis*) was statistically significantly associated with diarrhoea but appeared less pathogenic than *C. parvum* which was significantly associated with chronic diarrhoea and vomiting (Cama *et al.*, 2007). However, even when *Cryptosporidium* infections are not associated with diarrhoea, there is evidence that infection leads to a measurable effect on growth (Checkley *et al.*, 1997; Checkley *et al.*, 1998) and so it may be misleading to call non-diarrhoeic infection asymptomatic.

No previous work has evaluated the association between the cervine genotype and clinical manifestations, thus further work is required to determine the pathogenic potential of this genotype. The rabbit genotype was previously identified in an immunocompetent woman (no clinical symptoms reported) (Robinson *et al.*, 2008b) and in 23 patients with diarrhoea following a waterborne outbreak in the UK (Chalmers *et al.*, 2009a). It was subsequently identified as a human pathogen but since then no further research has been conducted.

Cryptosporidium is recognised as an important human pathogen especially in those with a compromised immune system, such as those with HIV/AIDS and the malnourished, and in paediatric populations. With the development of molecular tools for species identification and subtyping, more studies are required to evaluate the transmission routes and sources of infection for the various species, genotypes and subtypes, in addition to the pathogenic potential and public health significance of each.

3.4.4 Summary

A high diversity of *Cryptosporidium* species, genotypes and subtypes was isolated in this Nigerian paediatric population. *C. hominis* was the dominant species identified in 34 samples. *C. parvum* was the second most common infection (25 samples) followed by *C. meleagridis* (5 samples), *Cryptosporidium* rabbit genotype (5 samples), *Cryptosporidium* cervine genotype (3 samples) and *C. canis* (1 sample). Mixed infections of *C. hominis* and *C. parvum* were also identified in 4 samples. In addition to the isolation of a wide range of species, a number of subtypes of both *C. hominis* and *C. parvum* were identified highlighting the diversity of species and subtypes infecting these children. This high diversity may have implications for the clinical manifestations of disease as previous research has indicated that symptoms vary depending on the species and subtypes present. In addition, a high diversity of species and subtypes may indicate the presence of various sources of infection for the population but this was not determined epidemiologically.

Our data indicate that children can be the source of numerous *Cryptosporidium* species, genotypes and subtypes and that low hygiene and sanitation levels in the area are likely to result in both direct and indirect transmission routes for *Cryptosporidium*. The concurrent presence of human specific isolates, namely *C. hominis* and the *C. parvum* IIc subtype, suggest that transmission of infection may be through the anthroponotic rather than the zoonotic pathway in this population. However, further epidemiological investigations are required before the anthroponotic, zoonotic and / or environmental transmission routes of public health significance can be identified conclusively. Chapter 4 undertakes risk factor analysis to determine more clearly routes of transmission and sources of infection in this paediatric population in Nigeria.

4 Risk factors for sporadic *Cryptosporidium* infection

Section **Page**

Figure **Page**

Table **Page**

Plate **Page**

4.1 Introduction

Effective chemotherapeutic treatment for cryptosporidiosis has yet to be developed and so identifying risk factors for *Cryptosporidium* infection is essential in order to effectively reduce exposure to infectious oocysts. It is generally accepted that *Cryptosporidium* can be transmitted by a number of routes such as person-to-person contact, animal-to-person contact, consumption of contaminated drinking/recreational water and food and contact with contaminated environmental surfaces (Kosek *et al.*, 2001). The importance and significance of each transmission route for infection is likely to differ among regions and populations.

A number of studies, of either cross-sectional or longitudinal design, have been carried out in both the developed and the developing world to determine possible risk factors for *Cryptosporidium* infection. However, to date the majority of studies have focused on outbreak cases of cryptosporidiosis in developed countries (Osewe *et al.*, 1996; McAnulty *et al.*, 2000; Quiroz *et al.*, 2000; Stafford *et al.*, 2000; Causer *et al.*, 2006) and this lead to a paucity of data relating to risk factors for sporadic infection in general, and in particular within developing regions. Since risk factors are likely to vary between the developed and developing world, we urgently require risk factor analysis from tropical environments in order to better understand the transmission dynamics of *Cryptosporidium* under varying environmental and societal conditions.

Of the few studies carried out in developing countries, risk factors include younger age (under 2 years) (Khan *et al.*, 2004; Abdel-Messih *et al.*, 2005; Gatei *et al.*, 2006a), lack of breast-feeding (Molbak *et al.*, 1994; Bhattacharya *et al.*, 1997; Javier Enriquez *et al.*, 1997; Nchito *et al.*, 1998; Abdel-Messih *et al.*, 2005), contact with pets (Katsumata *et al.*, 1998; Xiao *et al.*, 2007b), living in over crowded conditions (Chacin-Bonilla *et al.*, 1997; Katsumata *et al.*, 1998; Newman *et al.*, 1999), low birth weight (Newman *et al.*, 1999), gender (Molbak *et al.*, 1994; Pereira *et al.*, 2002; Laubach *et al.*, 2004) and malnourishment (Bhattacharya *et al.*, 1997; Javier Enriquez *et al.*, 1997). However, the majority of these studies were based in Latin America with few conducted in sub-Saharan Africa (see Chapter 1, Table 1.6). To date, no study conducted in Nigeria has attempted to determine risk factors for infection.

Recently, evidence suggests that risk factors for *C. parvum* and *C. hominis* differ, therefore determining the species present for risk factor analysis may be particularly useful. Hunter *et al.* (2004) carried out a case-control study on sporadic cryptosporidiosis in patients in England and Wales. It was found that risk factors for infection included travel outside the UK, contact with another person with diarrhoea, touching cattle, helping a child <5 years to use the toilet and the number of glasses of tap water consumed each day. However, when risk factors were investigated separately for *C. parvum* and *C. hominis*, the main risk factors for *C. parvum* infection (contact with cattle) and *C. hominis* infection (travel abroad and changing diapers) differed. Although the power of a study is reduced when the analysis is restricted to cases where the species is known, when species are grouped, the analysis highlights factors common to both species, but downplays risk factors associated with one species or the other (Hunter *et al.*, 2004). Therefore, when carrying out such studies, it may be preferable to determine the species of *Cryptosporidium* causing the infections and to investigate *C. hominis* and *C. parvum* both together and separately for risk factor analysis.

This chapter addresses the following specific aims:

1. To determine the key socio-economic and other risk factors which are important in *Cryptosporidium* infection within a paediatric population in Nigeria

2. To determine the risk factors for *C. parvum* and *C. hominis* separately in order to determine whether risk factors vary between the species.

4.2 Materials and Methods

This cross-sectional study was conducted in August 2007, at the end of a longitudinal study which determined *Cryptosporidium* infection at 4 time points over a 1 year period (see Chapter 2). It is the first risk factor analysis for sporadic *Cryptosporidium* infection to be conducted in Nigeria.

4.2.1 Infection status

An infected individual was determined as a child that (1) attended the clinic in August 2007, (2) submitted a faecal sample for analysis, (3) completed a questionnaire and (4) tested positive for *Cryptosporidium* infection at this time. Infection was determined by the detection of oocysts in the stool using fluorescent microscopy (see Chapter 2, Section 2.2.4.3).

An uninfected individual was determined as a child that (1) attended the clinic in August 2007, (2) submitted a faecal sample for analysis, (3) filled out a questionnaire and (4) tested negative for *Cryptosporidium* infection at this time [as determined by the absence of oocysts in the stool sample determined by fluorescent microscopy (see Chapter 2, Section 2.2.4.3)].

A total of 735 children attended the clinic in August 2007. Of these, 692 submitted faecal samples and completed a questionnaire. Presence or absence of diarrhoea was not taken into consideration when choosing whether or not a child was infected. The aim of the current study was to determine risk factors for *Cryptosporidium* infection rather than cryptosporidiosis. Asymptomatic infection has been shown to be high in certain populations (Chacin-Bonilla *et al.*, 1993; Pettoello-Mantovani *et al.*, 1995; Esteban *et al.*, 1998; Houpt *et al.*, 2005; Palit *et al.*, 2005; Siwila *et al.*, 2007) and although the disease does not manifest in particular individuals, these asymptomatic hosts are sources of infection with the ability to spread and transmit *Cryptosporidium* to new susceptible individuals. Therefore, risk factors for infection are equally important for both asymptomatic and symptomatic hosts.

4.2.2 Questionnaires

Prior to the development of the questionnaire, a full literature review was carried out on risk factor studies for *Cryptosporidium* infection in order to determine the best approach to questionnaire design. Questionnaires were constructed taking into consideration the findings of previous studies in developing countries, especially in Africa, and including all relevant questions related to socio-economic status of the child and family and questions relevant to possible risk factors for infection in this particular area of Nigeria (see Appendix 3). The questionnaire was then revised by 3 knowledgeable colleagues: Professor Samuel Asaoulu of Obafemi Awolowo University, Ile-Ife, Nigeria; Professor Huw Smith from the Scottish Parasite Diagnostic Laboratory (SPDL), Glasgow and Dr Tracey Morse, an environmental health expert, who carried out her PhD on the epidemiology of *Cryptosporidium* in Malawi. The questionnaire was printed in English, and Yoruba-speaking interviewers translated the questions for the mothers and recorded their answers in English. The questionnaires were pre-tested (interviewers practiced on each other) and revised in Obafemi Awolowo University. All interviewers spoke both Yoruba and English, and were graduates of Obafemi Awolowo University, Ile-Ife, Nigeria.

4.2.3 Interviews

Prior to the beginning of fieldwork in August 2007, interviewers attended a training day and received a training document outlining how the questionnaires were to be administered (see Appendix 4). Each interviewer practiced administering the questionnaire to each other and any problems were highlighted and corrected. In addition, questionnaires and interviewers were monitored on the day of interviewing to ensure that no answers were missing and that the questionnaires were administered adequately.

In August 2007, clinics were running as part of the longitudinal study (see Chapter 2) and questionnaires were administered during these clinics to the mother of each child enrolled in the study (Plate 4.1). Stool samples were collected within 2 days post-questionnaire administration. Therefore neither the interviewer nor the interviewee was aware of which children were infected.

A parallel project running at this time assessed the prevalence of malaria and *Ascaris* infection in the same cohort of children (Kirwan *et al.*, 2009). Malaria infection was determined using a Parascreen rapid diagnostic test according to the manufacturer's instructions (RDTs; Zephyr Biomedicals, Verna Industrial Estate, Verna Goa, India) and stool samples were examined for *Ascaris* infection by means of the modified formal-ether concentration technique and subsequent microscopical analysis (Kirwan *et al.*, 2009).

Plate 4.1 Fieldworker administering a questionnaire to a child's mother
(Photo by: Síle Molloy)

4.2.4 Nutritional status

The nutritional status of each child enrolled in the study was assessed in August 2007 by measuring anthropometrics. The weight of each child was measured using an electronic balance (Plate 4.2) and height was measured using a stadiometer (Plate 4.3) (Awasthi and Pande, 2001). Using height and weight data, anthropometric indices i.e. z-scores for weight-for-age (WAZ), height-for-age (HAZ) and weight-for-height (WHZ) were calculated. A z-score is defined as the difference between the value for an individual and the median value of the reference population for the same age or height, divided by the standard deviation of the reference population (Cogill, 2003). The z-score system is considered the simplest way of describing the reference population and making comparisons to it.

Using the WHO system of classification (the most widely used system), z-scores of >-1 indicate healthy nutritional status, -1 to -2 indicate mild malnutrition, -2 to -3

indicate moderate malnutrition and z-scores of < -3 indicate severe malnutrition (Cogill, 2003). According to Cogill (2003), a low weight-for-age index identifies the condition of being underweight for a specific age. Low height-for-age index identifies stunting in the population and low weight-for-height identifies children suffering from wasting. Collection and analysis of anthropometric data allowed us to identify the nutritional status of this paediatric population and detect any association between *Cryptosporidium* infection and malnutrition.

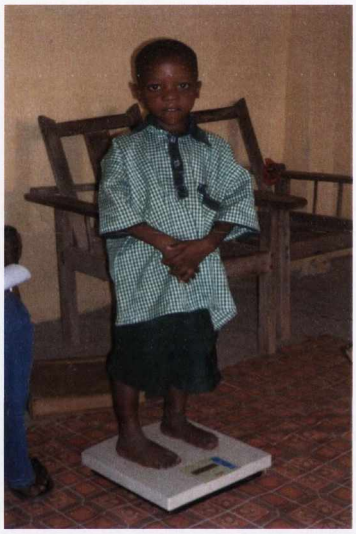

Plate 4.2 Weighing a child on an electronic balance
(Photo by: Síle Molloy)

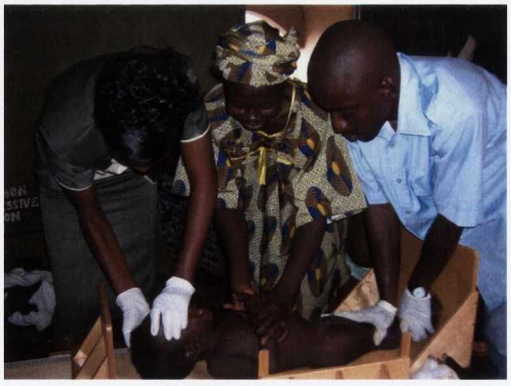

Plate 4.3 Measuring the height of an infant using a stadiometer
(Photo by: Síle Molloy)

4.2.5 Testing for *Cryptosporidium* oocysts and genotyping

Samples were tested for the presence of oocysts as described in Chapter 2 (Section 2.2.4.3). Likewise molecular techniques for determining the species and genotypes present were carried out as described in Chapter 3 (Section 3.2). The intensity of infection was recorded for each positive sample. Intensity was determined as follows: 0 = no oocysts detected in the sample; 1+ = 1-10 oocysts per field of view; 2+ = 10-50 oocysts per field of view and 3+ = >50 oocysts per field of view. The consistency of the stool sample was also noted and categorised into formed, unformed and watery.

4.2.6 Statistical analysis

Chi-squared analysis or Fisher's exact tests were used to test for an association between infection status and stool consistency and infection status and village of residence.

To determine which factors contribute to sporadic *Cryptosporidium* infection (presence/absense response), the data were fitted using a binomial generalised linear model (GLM) in R 2.9.0 (a software environment for statistical computing and graphics) (Crawley, 2007). A GLM is a type of linear model with a specialised error structure. The binomial family accounts for the way in which the outcome variable is described i.e. presence (1) or absence (0) of *Cryptosporidium*.

Multiple chi-squared tests were not carried out on the data prior to the development of the GLM (as previous studies have done) as a primary assumption of the chi-squared test is that it is the only test being carried out on the entire data set. Every time an additional test is conducted on the same group of data, the chance of finding some significant association increases. Thus, in the current Chapter, Section 4.3.1 of the results represents strictly descriptive and non-inferential data. Inferential statistics have been included in the GLM and risk factors for infection which were identified using this statistical analysis are presented in Section 4.3.2.

4.2.6.1 Indices

For data analysis, a number of indices were created in order to describe the population. These included a socio-economic status (SES) index, a hygiene index and an animal contact frequency index. Variables relating to each index were categorised, ranked and scored (Table 4.1). The scores allocated to the variables for each index were added, with a low index number indicating low socio-economic status, unhygienic practices and contact with few animals. When both the child's and mother's hygiene indices were created they were highly correlated (correlation coefficient = 0.377, $p<0.001$) and so an average of the 2 indices was used to describe the condition of parent and child.

Originally it was proposed that a food index be created but due to the structure of the questions in this part of the questionnaire and the fact that all individuals questioned wash their vegetables before eating, the construction of this index was not possible. Many of the questions were correlated and depended on the answer of a previous question e.g. Is water added to the milk? If so, do you treat the water? The latter question could not be used as it was dependent on the answer to the previous question. Exclusive breast-feeding was included in the main model as this was considered the most important variable in the food section of the questionnaire.

Likewise, the creation of a water index was proposed. However, as for the food index, the structure of the questions in this part of the questionnaire did not allow for the creation of a valuable index. For treatment of water, the method and frequency of treatment were determined [e.g. boiling / filtering - always, never or sometimes (see Appendix 3)]. These were to be ranked and scored but it was not possible to decide whether, for example, boiling the water *sometimes* was better than *always* filtering the water or *always* allowing it to settle. Therefore, boiling of water before drinking was included in the model as boiling was considered the most important method of water treatment for removing *Cryptosporidium* and if the water was boiled, the source of the drinking water becomes less important.

Table 4.1 Construction of socio-economic, hygiene and animal contact frequency indices

Index	Variables	Score (ranking)
Socio-economic index	No. of possessions	Fridge = 1 Generator = 1 Television = 1 Radio = 1
	Fuel used in the household	Firewood = 1 Gas/Kerosene = 2 Electricity = 3
	Number of mobile phones	None = 0 Father OR mother = 1 Father AND mother = 2
	Mother's occupation	Housewife, manual worker, student, deceased = 1 Farmer, craftswoman, business woman, street vendor = 2 Civil servant, professional, clerk, teacher = 3
	Father's occupation	Househusband, manual worker, student or deceased = 1 Farmer, craftsman, businessman, street vendor, driver = 2 Civil servant, professional, clerk, teacher, pastor = 3
	Level of income	≤4,999N* = 1 5,000-9,999N = 2 ≥10,000N = 3
Child's hygiene index	Method of hand washing	Wash hands before eating = 1 Wash hands after going to the toilet = 1 Using soap or soda to wash the hands = 1 Use a tap or, cup and water, to wash hands = 1 Air drying = 1
	Type of toilet used	Bush, potty or the ground = 1 Pit latrine = 2 Flush toilet = 3

* N = Nigerian Naira

Table 4.1 Contd

Index	Variables	Score (ranking)
Mother's hygiene index	Method of hand washing	Wash hands before eating = 1 Wash hands after going to the toilet = 1 Wash hands after feeding the child = 1 Using soap or soda to wash the hands = 1 Use a tap or, cup and water, to wash hands = 1 Air drying = 1
	Type of toilet used	Bush, potty or the ground = 1 Pit latrine = 2 Flush toilet = 3
Animal contact frequency index	No. of animals the child had contact with	Dog = 1 Cat = 1 Goat = 1 Chicken = 1

4.2.6.2 Additional variables

Metrics describing each child's nutritional status were also recorded. These included weight-for-age, height-for-age and weight-for-height. The weight-for-height metric was highly correlated to both height-for-age (correlation coefficient = 0.367, $p < 0.001$) and weight-for-age (correlation coefficient = 0.271, $p < 0.001$), but weight-for-age and height-for-age were not correlated (correlation coefficient = 0.011, $p = 0.777$). Therefore, weight-for-height was excluded from the analysis.

The child's clinical symptoms were not included in the model as these are the consequences of an infection i.e. an outcome rather than a risk factor.

4.2.6.3 Generalised linear models

The initial full model included the following explanatory variables:
age of the child (months), gender, the level of maternal education [coded as 0 (none), 1 (primary), 2 (secondary), 3 (tertiary)], height-for-age [coded as 0 (healthy), 1 (mildly), 2 (moderately) and 3 (severely stunted)], weight-for-age [0

(healthy), 1 (mildly), 2 (moderately) and 3 (severely underweight)], exclusive breast-feeding (children breast-fed exclusively until 6 months of age), socio-economic status, animal contact frequency index, hygiene index, household crowding (i.e. number of people per room), boiling of drinking water, diarrhoeal status of members of the household, *Ascaris* infection, and malaria infection. The socio-economic index was significantly correlated with the level of maternal education (correlation coefficient = 0.450, $p < 0.001$), hygiene index (correlation coefficient = 0.128, $p < 0.001$) and animal index (correlation coefficient = -0.147, $p < 0.001$). Therefore, GLM models in which the full model included either socio-economic index, level of maternal education, hygiene index, or animal index were all analysed independently, and the best fitting model was retained. The final model was chosen according to a combination of parsimony and model fit (explanatory power of the remaining variables) based on Akaike's information criterion (AIC) scores (Sakamoto *et al.*, 1986). This is a measure of the goodness-of-fit estimated by the statistical model. The model with the lowest AIC value represents the best fit model to explain the observed data.

In addition to analysing these factors independently in a full model context, the interaction of certain factors regarding *Cryptosporidium* infection was determined. This *post hoc* analysis was performed to investigate how particular factors might interact with 1 another in ways that might mask the effect of either 1 or both factors (Crawley, 2007).

Molecular analysis for the determination of species was conducted on all positive samples (134 samples) collected in August 2007 (see Chapter 3), however, of these, 49 (36.6%) produced sufficient product for RFLP determination of species. Therefore, species data for 49 samples were available for risk factor analysis. Because infected individuals could have any number of different types of *Cryptosporidium* infections (and therefore variations in risk factors for infection), 3 additional GLM analyses were created to determine whether risk factors differed for *C. hominis* and *C. parvum*. The first analysis incorporated all samples that were either *C. hominis* or *C. parvum*. The second incorporated samples that were *C. hominis* only and the third *C. parvum* only. These analyses were performed using the same approach as that described above for the full model. Interactions between explanatory variables and infection could not be explored due to the small sample sizes of *C. hominis* and *C. parvum*-infected populations.

A total of 692 questionnaires were filled out during August 2007 and these were entered into STATA, Version 10.0 (Rabe-Hesketh *et al.*, 2002). Data were validated using consistency checks and any records with errors were corrected from the original questionnaire and re-entered to produce the final data set. The data were transferred from STATA and statistical analysis was performed using R 2.9.0 (Crawley, 2007).

4.3 Results

4.3.1 Overview

During August 2007, 735 children submitted stool samples for analysis and 692 questionnaires were completed. Therefore 692 children were included in the analysis as both infection status and information from the questionnaire were required for each child. In order to give a general overview of the lives of the people living in this area of Nigeria, the results from the questionnaires follow for all 692 children (134 infected children; 558 uninfected children).

4.3.1.1 Age, gender and village of residence

Of the 692 children, 148 (21.4%) were from the village of Akinlalu, 298 from Ipetumodu (43.1%), 84 from Moro (12.1%) and 162 from Edunabon (23.4%) (Figure 4.1). Almost equal numbers of females (357) and males (335) attended (Table 4.2) and ages of the children ranged from 19.5 to 72 months (median of 43.7 months). However, few children (40) were under 2 years of age (Figure 4.2).

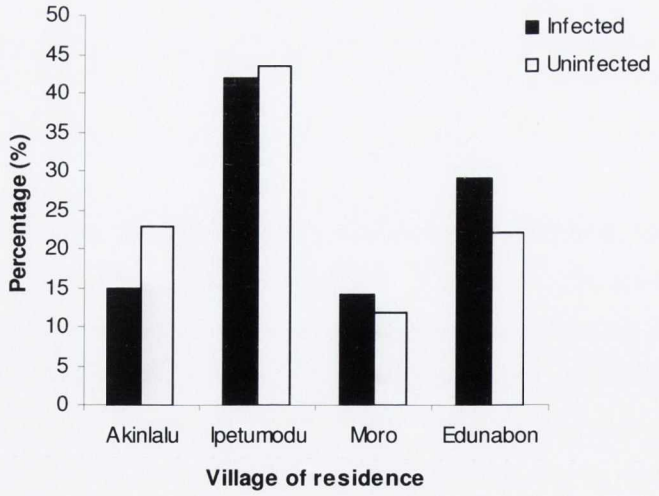

Figure 4.1 Distribution of infected and uninfected children living in each of the 4 villages

Table 4.2 Distribution of infected and uninfected children by gender

	Gender	
	Female (%)	**Male (%)**
Infected	61 (45.5)	73 (54.5)
Uninfected	296 (53)	262 (47)
Total	357 (51.6)	335 (48.4)

Figure 4.2 Age distribution of infected and uninfected children

4.3.1.2 Intensity and stool consistency

The majority of infections were of low intensity (88.8%, 1+), while 2.3% were medium intensity (2+) infections and 8.2% high intensity (3+). Stool consistency was recorded in the laboratory and divided into 3 categories: formed, unformed and watery. Overall, 203 children had formed stools, 469 had unformed stools and 20 watery stools (Table 4.3). When analysed using chi-squared analysis, there was a significant association between the stool consistency and infection status (χ^2=6.04; df=2; p=0.049) implying that infection is mildly associated with watery stools. However, it must be noted that the number of watery samples collected was very low (20).

Table 4.3 Stool consistency of infected and uninfected children

	Stool consistency		
	Formed (%)	**Unformed (%)**	**Watery (%)**
Infected	35 (26.1)	91 (67.9)	8 (6.0)
Uninfected	168 (30.1)	378 (67.7)	12 (2.2)
Total	203 (29.3)	469 (67.8)	20 (2.9)

4.3.1.3 Education and occupation

The majority of both fathers (62.7%) and mothers (60.1%) were educated to secondary level and very few had no education at all (4.2%) (Table 4.4). Most children attended school (66.7%) while fewer attended day care (18.1%) and fewer still stayed at home (15.2%). The majority of mothers were business women (53.3%) or craftspeople (20.6%) with few women working as clerks (0.3%) or working solely in the home (0.7%). In contrast, fathers were most commonly farmers (25.3%) and craftspeople (24.9%). Few men worked solely in the home (0.3%) or as clerks (0.9%) and a number of fathers were deceased (0.9%).

There was a range of monthly income; 1,000N-60,000N with an average of 11,000N (N=Naira; Nigerian currency). The majority of families earned between 5,000N and 10,000N (48.7%) (Table 4.5).

4.3.1.4 Housing

The number of people living in each house ranged from 2-48 people with a median of 12. For infected children the number of people living in the house ranged from 3-48, similar to the range for uninfected children (2-47), however, the median was much higher in households with infected children (14) in comparison to those with uninfected children (7). The number of adults per household ranged from 1-25 (median 6), the number of children aged between 5 and 15 years from 0-17 (median 3) and those less than 5 years from 0 - 32 (median 3). The range and median number of adults and children aged 5-15 years in each household was similar for both infected (0-15) and uninfected (0-17) children. However, a greater range in the number of children less than 5 years of age was observed in households with infected

children (0-32, median 5) in comparison to those with uninfected children (0-10, median 3).

Houses were often shared by a number of families (up to 30, median of 3) and some houses were large with up to 22 rooms (median 6). A maximum of 3 people per room was observed (median of 0.55 people per room) and similar results were obtained for households with both infected (median of 0.57 people per room) and uninfected (median of 0.55 people per room) children.

Table 4.4 Parent's level of education for infected and uninfected children

	Maternal education level (%)				Paternal education level (%)			
	None	**Primary**	**Secondary**	**Tertiary**	**None**	**Primary**	**Secondary**	**Tertiary**
Infected	7 (5.2)	32 (23.9)	79 (59.0)	16 (12.0)	1 (0.8)	11 (8.3)	95 (71.4)	25 (18.8)
Uninfected	22 (3.9)	128 (23.0)	337 (60.4)	71 (12.7)	13 (2.4)	64 (11.6)	335 (60.9)	138 (25.0)
Total	29 (4.2)	160 (23.1)	416 (60.1)	87 (12.6)	14 (2.0)	75 (11.0)	430 (62.8)	163 (23.8)

Table 4.5 Family income for infected and uninfected children

	Monthly income group (%)		
	<5,000N	**5-10,000N**	**>10,000N**
Infected	31 (23.9)	60 (46.1)	39 (30.0)
Uninfected	101 (18.5)	269 (49.3)	176 (32.2)
Total	132 (19.5)	329 (48.7)	215 (31.8)

4.3.1.5 Socio-economic status

Few families owned generators (15.8%) or fridges (33%) but many had radios (97.3%) and televisions (87.4%). The most common fuels used in these areas were gas (49.6%) and kerosene (45.5%) with few families using electricity (4.3%) or firewood/coal (1%). 79% of families had at least 1 mobile phone.

An SES index was constructed as described in Section 4.2.6.1. and ranged from 8 to 17 with a median of 12 (Figure 4.3)

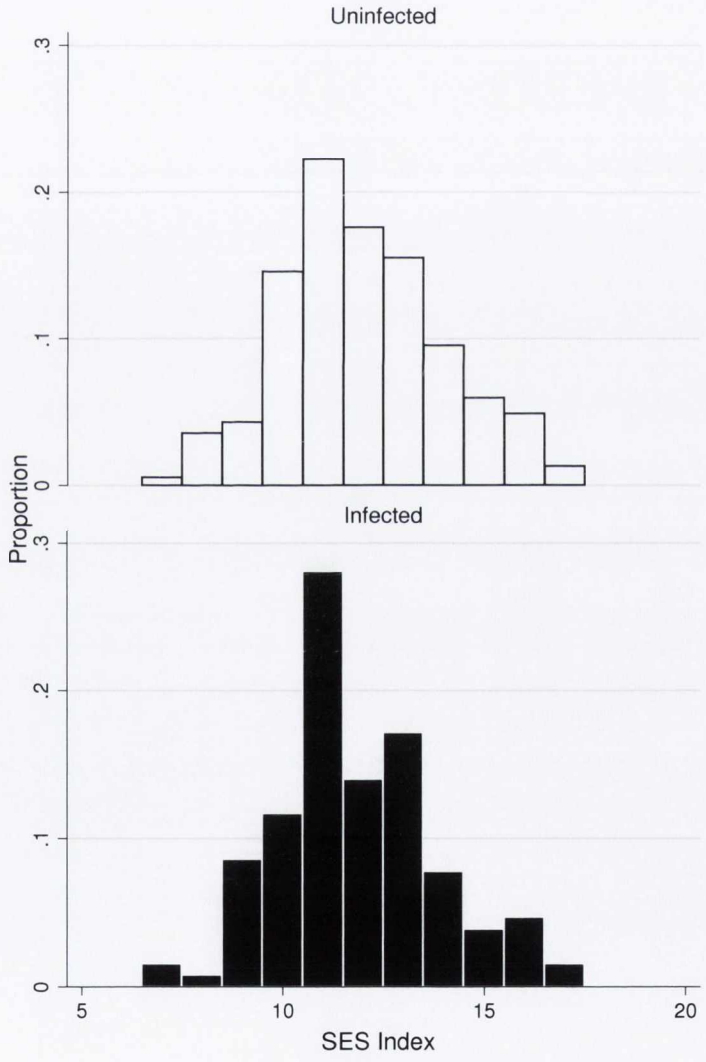

Figure 4.3 Distribution of socio-economic indices of the households of uninfected and infected children

4.3.1.6 Water

The people of this area used multiple sources of water for drinking (Figure 4.4). Municipal water supply from taps located within houses or in the community (Plate 4.4) was the most common source of drinking water (80.2%), followed by the use of well water (27.5%) (Plate 4.5) and rain water (26.5%). Few people used boreholes (2%) (Plate 4.6) or river water (2.7%) and less than half of the families treated their water in some way (Table 4.6). Of those that used treatment most boiled the water (57%) and/or allowed the water to settle (65%). Chlorination, the addition of alum (a flocculent), and filtering were less common methods of water treatment in this area.

Children generally bathed in outdoor showers (36.6%) (Plate 4.7) or used buckets and water inside the house (61.6%) and most obtained well water for bathing (75%).

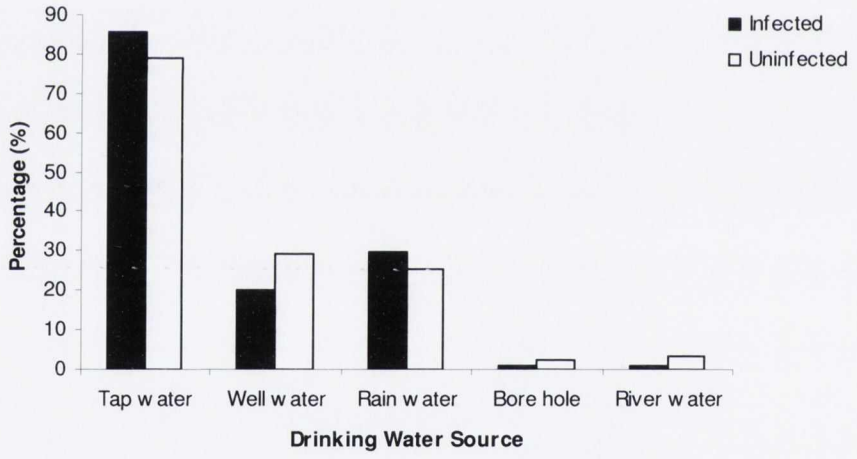

Figure 4.4 Percentage of respondents using various drinking water sources

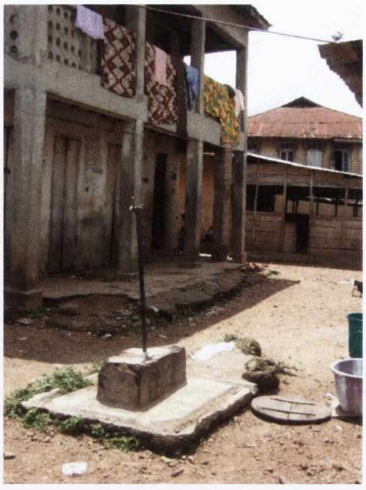

Plate 4.4 Municipal water supply from a community tap
(Photo: Síle Molloy)

Plate 4.5 Mother collecting drinking water from a community well
(Photo: Síle Molloy)

Plate 4.6 Boreholes within the community (Photo: Síle Molloy)

Plate 4.7 Outdoor shower beside family home (Photo: Síle Molloy)

Table 4.6 Drinking water treatment for infected and uninfected children

	Water treatment (%)	
	Yes	**No**
Infected	64 (47.8)	70 (52.2)
Uninfected	253 (45.3)	305 (54.7)
Total	317 (45.8)	375 (54.2)

4.3.1.7 Hygiene

The majority of adults used pit latrines as toilets (56%) (Plate 4.8) or went outside in the bush (26.6%). Few families had flush toilets (16.6%). Children, on the other hand, mostly used a potty (80.9%) (Table 4.7) and no child was still in nappies.

Plate 4.8 Pit latrine used by a number of houses in Akinlalu
(Photo: Síle Molloy)

Table 4.7 Percentage of mothers and children that used various toilet types by infected and uninfected children

	Mother (%)			Child (%)		
Toilet type	**Infected**	**Uninfected**	**Total**	**Infected**	**Uninfected**	**Total**
Flush Toilet	25 (18.8)	90 (16.1)	115 (16.6)	6 (4.5)	11 (2.0)	17 (2.5)
Pit latrine	68 (51.1)	319 (57.2)	387 (56.0)	3 (2.3)	7 (1.3)	10 (1.5)
Bush/Potty	40 (30.1)	149 (26.7)	189 (27.4)	124 (93.4)	540 (96.8)	664 (96.1)

Adults most commonly washed their hands before eating (84.7%), after eating (52.2%) and after using the toilet (59.6%) while very few washed their hands before feeding the child (1.2%). Similarly, children washed their hands before eating (84.9%), after eating (49.4%) and less commonly after using the toilet (37%) (Table 4.8). 46.5% of adults used water only to wash the hands while the remaining 53.5% used water and soap or water and soda. The most common method for adults to wash their hands was using a bowl filled with water (84%), while the method of filling a cup with water and pouring on the hands was also utilised (16.1%). Methods of hand washing did not vary between infected and uninfected children, or between

mothers of infected and uninfected children. A towel/rag was used to dry the hands (81.3%) in the majority of cases while some air dried. No important differences were observed between mothers with infected or uninfected children. Children generally followed the same pattern as the adults in relation to the methods of washing and drying hands.

The hygiene index ranged from 2 to 8.4 with a median of 4 (Figure 4.5). A high hygiene index indicated good hygiene practices.

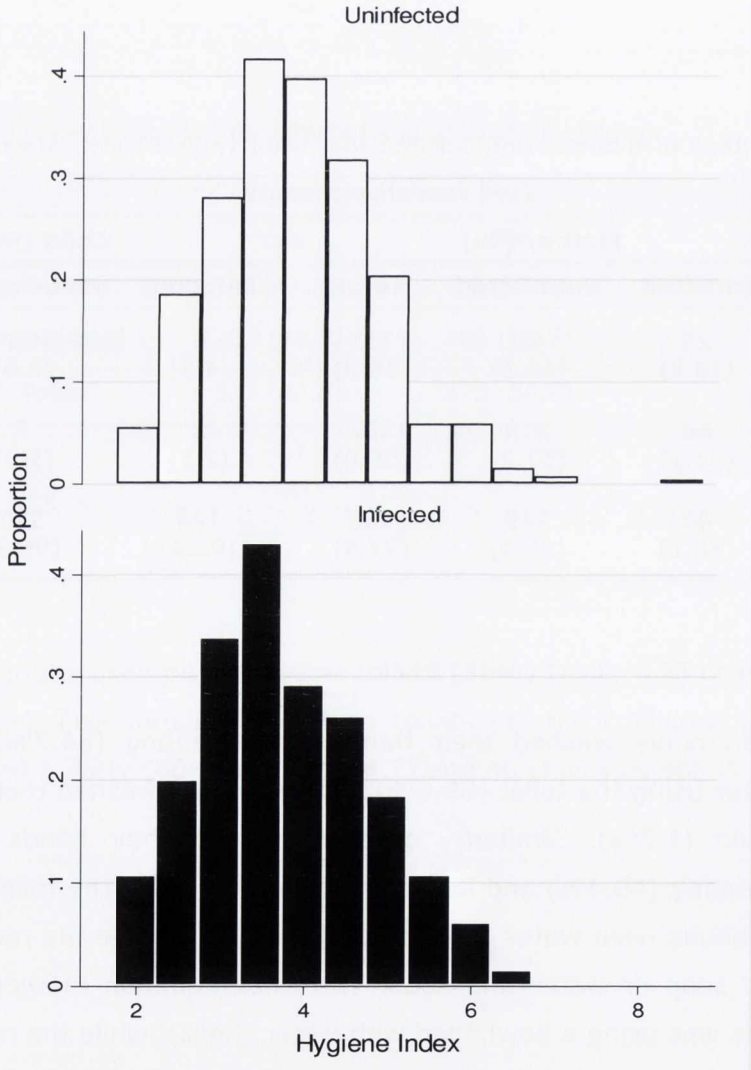

Figure 4.5 Distribution of the hygiene index for mothers and children by uninfected and infected children

Table 4.8 Times when mothers and children washed their hands by infected and uninfected children

Time	Mother (%)			Child (%)		
	Infected child	Uninfected child	Total	Infected	Uninfected	Total
Before meal	113 (84.3)	472 (84.7)	585 (84.7)	109 (82.0)	478 (85.7)	587 (85.0)
After meal	71 (53.0)	290 (52.0)	361 (52.2)	69 (51.9)	272 (48.8)	341 (49.4)
After toilet use	74 (55.2)	338 (60.7)	412 (59.6)	48 (36.1)	208 (37.3)	256 (37.1)
Before cooking	25 (18.7)	70 (12.6)	95 (13.8)	N/A	N/A	N/A
When dirty	8 (6.0)	44 (7.9)	52 (7.5)	14 (10.5)	71 (12.7)	85 (12.3)
After work/play	15 (11.2)	88 (10.4)	73 (10.6)	(21.1)	74 (13.3)	102 (14.8)
Before feeding child	2 (1.5)	6 (1.1)	8 (1.2)	N/A	N/A	N/A

4.3.1.8 Food

Generally tinned (58.1%) or powdered milk (36.3%) were given to the children. Soya milk (2.3%), fresh cow's milk or goat's milk (1.7%) were seldom consumed. Water was often added to milk (89.8%) but in many cases (38%) this water was not treated.

Very few children were breast-fed at the time of questioning (5). This probably reflects the older age of the children. Generally children had been breast-fed up to the age of 20 months and 75.6% of children (76.9% of infected children and 70.2% of uninfected children) had been breast-fed exclusively for an average of 6 months. Children were, on average, 4.4 months old when first given water and 10.9 months when given solids for the first time.

The majority of families bought their food at the market (77.3%) while some grew their own (9.4%) and some did both (13.3%). When vegetables were grown, river water was most commonly used for irrigation (35.4%) followed by rain water (32.9%) and water from boreholes (20.3%). Neither chemicals (13.9%) nor animal manure (3.2%) were commonly used as fertilisers and no human waste was applied to the land.

All people interviewed washed the vegetables before eating (100%) but only 26.8% treated the water used for washing (31.3% of infected children and 25.3% of uninfected children). Fruit and vegetables were generally washed using well water (53.8%) or tap water (41.3%).

4.3.1.9 Animals

The majority of families (86.3%) owned animals (91.6% of families with infected children kept animals compared with 85.0% of families with uninfected children). Goats (62.9%) and chickens (80.5%) (Plate 4.9 and 4.10) were most frequently kept while ducks and sheep were much less common (7.2% combined) (Table 4.9). No family kept cows or rabbits nor did they note that their children had contact with either of the two.

Plate 4.9 Cages for keeping chickens in the yard of a house
(Photo: Síle Molloy)

Plate 4.10 Goats and chickens roaming freely in the yards of the houses
(Photo: Síle Molloy)

Table 4.9 Percentage of families that kept various types of animals

	Families keeping animals (%)		
Animals	**Infected**	**Uninfected**	**Total**
Dog	28 (20.9)	94 (16.9)	122 (17.6)
Goat	93 (69.4)	342 (61.3)	435 (62.9)
Cow	0	0	0
Cat	12 (9.0)	73 (13.1)	85 (12.3)
Chicken	114 (85.1)	443 (79.4)	557 (80.5)
Other	15 (11.2)	35 (6.3)	50 (7.2)

4.3.1.10 Health status of children

Three indicators were used to assess the nutritional status of the children. Height-for-age indicated the level of stunting experienced by a child, weight-for-age indicated whether a child was under weight for a specific age and weight-for-height indicated the level of wasting experienced by a child. The majority of children were either healthy or mildly malnourished (Table 4.10). A higher proportion of children were severely stunted (6.1%) than those severely underweight (2.6%) or wasted (0.9%).

Table 4.10 Nutritional status of infected and uninfected children

	Nutritional status (%)			
	Healthy	**Mild**	**Medium**	**Severe**
Stunting				
Infected	48 (36.4)	44 (33.3)	32 (24.2)	12 (8.8)
Uninfected	233 (41.8)	192 (34.5)	98 (17.6)	30 (5.4)
Total	281 (40.8)	236 (34.3)	130 (18.9)	42 (6.1)
Underweight				
Infected	46 (34.6)	62 (46.6)	21 (15.8)	4 (3.0)
Uninfected	216 (38.8)	233 (41.8)	94 (16.9)	14 (2.5)
Total	262 (38.0)	295 (42.8)	115 (16.7)	18 (2.6)
Wasting				
Infected	95 (72.0)	29 (22.0)	7 (5.3)	1 (0.8)
Uninfected	396 (71.4)	136 (24.5)	18 (3.2)	5 (0.9)
Total	491 (71.5)	165 (24.0)	25 (3.6)	6 (0.9)

4.3.1.11 Ascaris and malaria infection

Data for both *Ascaris* and malaria infections were collected by Dr Patrick Kirwan during a parallel study set up in the area which assessed the interaction between *Ascaris* and malaria infection in the same cohort of children (Kirwan *et al.*, 2009). These data were obtained from Dr Kirwan for the children in the current study, and any associations between *Cryptosporidium* infection and *Ascaris* and malaria were

investigated. *Ascaris* data were available for a total of 668 children while malaria data were available for 691 children (Table 4.11 and 4.12).

Table 4.11 Number of children infected with *Ascaris* and *Cryptosporidium*

	***Ascaris* infection (%)**	
***Cryptosporidium* infection**	**Infected**	**Uninfected**
Infected	24 (18.5)	106 (81.5)
Uninfected	166 (30.9)	372 (69.1)
Total	190 (28.4)	478 (71.6)

Table 4.12 Number of children infected with malaria and *Cryptosporidium*

	Malaria infection (%)	
***Cryptosporidium* infection**	**Infected**	**Uninfected**
Infected	103 (76.9)	31 (23.1)
Uninfected	379 (68.0)	178 (32.0)
Total	482 (69.8)	209 (30.2)

4.3.1.12 Species and genotypes

All 134 positive stool samples were analysed using 2 nested PCR-RFLP and / or direct sequencing of PCR products. Of these, 49 samples produced sufficient product for RFLP determination of species. Where required, DNA sequencing was used to confirm *Cryptosporidium* species / genotypes. *C. hominis* accounted for 37.3% (19) samples, 35.3% (18) *C. parvum* samples were isolated and there were mixtures of the 2 species in 7.8% (4) of samples. Other species isolated included, *Cryptosporidium meleagridis* (4, 7.8%), *Cryptosporidium* rabbit genotype (2, 3.9%), *Cryptosporidium* cervine genotype (1, 2.0%) and *Cryptosporidium canis* (1, 2.0%) (Table 4.13). The distribution of *C. hominis* and *C. parvum* by age, gender and village is presented in Table 4.14.

Table 4.13 Species of *Cryptosporidium* isolated from a Nigerian paediatric population

***Cryptosporidium* species**	**No. (%)**	**Subtype families (No.)**
C. hominis	19 (37.3)	Ia (9), Ib (6), Id (3)
C. parvum	18 (35.3)	IIa (1), IIc (14), IIi (1), IIm (2)
C. parvum/C. hominis	4 (7.8)	
C. meleagridis	4 (7.8)	
Rabbit genotype	2 (3.9)	
Cervine genotype	1 (2.0)	
C. canis	1 (2.0)	
Total	49 (100)	

Table 4.14 *C. parvum* and *C. hominis* distribution by age, gender and village

	***Cryptosporidium* species**	
Variable	***C. parvum* (%)**	***C. hominis* (%)**
Age		
≤ 2 yrs	2 (11.1)	1 (5.3)
2-4 yrs	9 (50)	14 (73.7)
≥ 4 yrs	7 (38.9)	4 (21.1)
Gender		
Male	9 (50)	11 (57.9)
Female	9 (50)	8 (42.1)
Village		
Akinlalu	4 (22.2)	2 (10.5)
Ipetumodu	9 (50)	7 (36.8)
Edunabon	1 (5.6)	3 (15.8)
Moro	4 (22.2)	7 (36.8)

4.3.2 Generalised linear models

To determine whether the prevalence of *Cryptosporidium* infection differed among the villages, infection counts were analysed using chi-squared or Fisher's exact tests. No association was detected between *Cryptosporidium* infection and village of residence [all *Cryptosporidium* infections (χ^2=6.18, df=3, p=0.103) individuals with either *C. hominis* or *C. parvum* (Fisher's exact test, p=0.771), only individuals with *C. hominis* (Fisher's exact test, p=0.3847), and only those with *C. parvum* (Fisher's exact test, p=0.918)], thus data from all the villages were combined for risk factor analysis.

The final overall model for *Cryptosporidium* infection (n=692, 134 positive samples) included the variables presented in Table 4.15 (AIC = 625.8). Risk of *Cryptosporidium* infection decreased for children with *Ascaris* infection and a trend was observed towards a decreased risk for those who had been breast-fed exclusively for the first 6 months of life. A trend for increased risk of *Cryptosporidium* infection for children infected with malaria was also observed. Furthermore, the interaction between crowding and a member in the household with diarrhoea was statistically significant (Table 4.15, Figure 4.6) indicating that when household crowding is low, the presence or absence of a member with diarrhoea in the household has no significant effect on the probability of a child becoming infected. However, as density increases, a child living in a household with at least 1 individual suffering from diarrhoea is much more likely to be infected with *Cryptosporidium* than a child in a house with a high density of people and no individuals suffering from diarrhoea. Neither household crowding nor living with an individual with diarrhoea were significant as main effects.

Table 4.15 Variables included in the final model for all *Cryptosporidium* infections

Variable	Coefficient	p value
Household crowding	0.0009	0.997
Member in household with diarrhoea	-2.277	0.097
Ascaris infection	-0.718	0.003
Malaria infection	0.389	0.072
Exclusive breastfeeding	-0.352	0.075
Household crowding * Member in household with diarrhoea	4.453	0.028

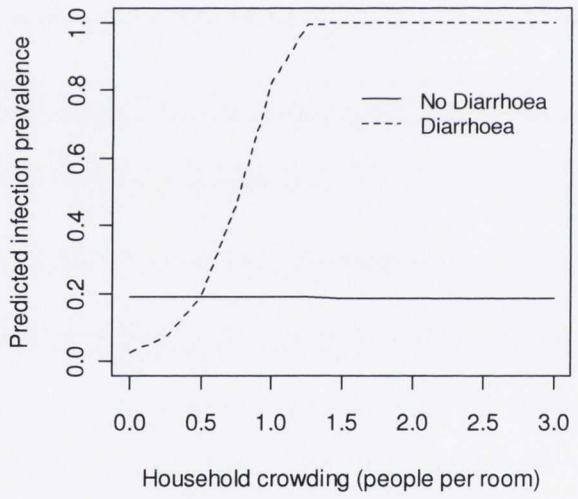

Figure 4.6 GLM (family=binomial) model fit predictions of the data showing interactions between diarrhoea and household crowding.

The final model for infection with either *C. hominis or C. parvum* (n=692, 37 positive samples) included the variables presented in Table 4.16. Variables for the final model of infections with *C. hominis* only (n=692, 19 positive samples) and *C. parvum* only (n=692, 18 positive samples) are presented in Tables 4.17 and 4.18, respectively.

For children infected with either *C. hominis* or *C. parvum* (Table 4.16), risk factors for infection included younger age, malaria infection, decreased level of maternal

education (from tertiary to none) and increased severity of stunting (from a healthy height-for-age to severe stunting).

For *C. hominis* infections alone (Table 4.17), younger age was statistically significantly associated with risk of infection, while there was a trend for an increased risk of infection with decreasing levels of maternal education and increased severity of stunting.

Furthermore, for children infected with *C. parvum* only (Table 4.18), increased severity of stunting and malaria infection were identified as significant risk factors for infection.

Although height-for-age was not highlighted as a risk factor in the main model, it was statistically significant in the model including both *C. hominis* and *C. parvum* (p=0.005) and in the *C. parvum* model (p=0.048) with a trend towards significance observed in the *C. hominis* model (p=0.066). Similarly, age of the child was not a risk factor in the main model or in the *C. parvum* model but was a significant risk for children infected with either *C. hominis* or *C. parvum* (p=0.015) and for those infected with *C. hominis* only (p=0.036).

The level of maternal education was not highlighted as a risk factor in the main model or in the *C. parvum* model but was significantly associated with risk in children infected with either *C. hominis* or *C. parvum* (p=0.041) and a trend towards significance was observed in *C. hominis* infected children (p=0.060).

Ascaris infection was negatively associated with risk in the main model (p=0.003) but no association was observed when the model was broken down by species. Malaria infection showed a trend towards increased risk of infection in the main model (p=0.072) and in the *C. parvum* model (p=0.068), was statistically significantly associated with risk in the model with *C. parvum* and *C. hominis* but was not implicated as a risk factor when *C. hominis* was considered separately.

Table 4.16 Variables included in the final model for *C. hominis* or *C. parvum* infections (AIC = 274.9)

Variable	Coefficient	p-value
Height-for-age	0.394	0.005
Age of the child	-0.031	0.015
Level of maternal education	-0.453	0.041
Malaria infection	0.968	0.045

Table 4.17 Variables included in the final model for *C. hominis* infections (AIC = 171.2)

Variable	Coefficient	p-value
Height-for-age	0.346	0.066
Age of the child	-0.039	0.036
Level of maternal education	-0.572	0.060

Table 4.18 Variables included in the final model for *C. parvum* infections (AIC = 160.1)

Variable	Coefficient	p-value
Height-for-age	0.42	0.048
Malaria infection	1.88	0.068

4.4 Discussion

This is the first study to identify risk factors for sporadic *Cryptosporidium* infection in Nigeria and the first in Africa to consider risk factors separately for *C. hominis* and *C. parvum* species. Furthermore, only 3 studies in 2 other geographical locations in developing countries have identified risk factors for infection by species and these were conducted in Peru and India (Ajjampur *et al.*, 2007; Cama *et al.*, 2007; Cama *et al.*, 2008).

4.4.1 Epidemiological methods

The study design adopted by researchers for epidemiological analysis of risk factors for *Cryptosporidium* infection varies considerably. Studies may be longitudinal, such as a cohort study, where healthy participants are recruited and followed over time to determine which individuals develop the disease, or cross-sectional, where a representative sample of individuals is assessed for the presence of disease (at a particular point in time) and their status with regard to risk factors for infection is examined, usually by means of a questionnaire. In addition, case-control studies [which can be longitudinal (prospective) or cross-sectional (retrospective)] select case patients and controls and their exposure to varying environmental conditions are compared. Furthermore, the definition of a case in case-control studies varies.

Case-control studies generally use both clinical symptoms and laboratory confirmation of infection to define a case. However, the methods for the detection of oocysts in the stool differ among studies, as does the determination of clinical symptoms. Some studies define a case as those that are *Cryptosporidium* positive and suffering from diarrhoea at the time of questioning (Bhattacharya *et al.*, 1997; Goh *et al.*, 2004), while others recruit cases that had suffered from diarrhoea within 2 weeks of data collection (Hunter *et al.*, 2004) and in Australia, a case was defined as a *Cryptosporidium* positive individual that had any vomiting or diarrhoea within 8 weeks before stool analysis and issuing questionnaires (Robertson *et al.*, 2002). In 2008, the Centre for Disease Control (CDC) proposed 2 definitions of a case for *Cryptosporidium*: (1) confirmed, symptomatic: a laboratory-confirmed case

associated with 1 of the following symptoms; diarrhoea, abdominal cramps, loss of appetite, low-grade fever, nausea or vomiting and (2) confirmed, asymptomatic: a laboratory-confirmed case associated with none of the above symptoms. Case-control studies should endeavour to standarise the definition of a case in accordance with the CDC in order to make comparisons between studies more efficient.

Case-control studies are often used to assess risk factors for outbreaks of *Cryptosporidium* infection in developed regions (Osewe *et al.*, 1996; McAnulty *et al.*, 2000; Quiroz *et al.*, 2000; Stafford *et al.*, 2000; Causer *et al.*, 2006). Of the few case-control studies conducted in developing countries, all have focused on risk factors for sporadic *Cryptosporidium* infection (Molbak *et al.*, 1994; Bhattacharya *et al.*, 1997; Khan *et al.*, 2004) (Table 4.19) and so this has lead to a paucity of data relating to outbreaks of cryptosporidiosis in the developing world, probably owing to a lack of surveillance.

The majority of studies conducted in developing countries, however, have adopted the cross-sectional study design to determine risk factors for sporadic *Cryptosporidium* infection, collecting stool specimens from individuals and using data from questionnaires to determine risk factors for infection (Table 4.19). However, within the cross-sectional study design, there are significant variations with respect to the following factors: sample size, time of year, age group, diarrhoeal status, immune status, laboratory methods for diagnosis and statistical analysis, making comparisons between studies problematic. Table 4.19 highlights the differences in design between risk factor studies conducted in developing countries. Although comparing results may be helpful in order to extrapolate potential risk factors to regions that have not yet been investigated, one should always be aware of the differences in study design and statistical analysis between the studies.

The present study adopted a cross-sectional design whereby each child was sampled on 1 occasion and data relating to risk factors were collected by means of a questionnaire. Data were collected from all children attending the clinic and infection status was not determined until after data collection. Therefore, an important source of bias was removed from the data, as neither the mother nor the interviewer was aware of which child was infected. From a statistical point of view, our study used a robust generalised linear model (GLM), incorporating a range of variables, to assess a representative sample of the population. Therefore, results can potentially be

extrapolated to all children in this age group within the population. In addition, we assessed risk factors for sporadic *Cryptosporidium* infection rather than sporadic cryptosporidiosis. It is important to understand risk factors for both symptomatic and asymptomatic hosts as research has indicated that *Cryptosporidium* infection can have lasting adverse effects on growth, even in children without diarrhoea (Checkley *et al.*, 1997; Checkley *et al.*, 1998). Also, asymptomatic hosts can act as a source of infection for more susceptible individuals in the population and so transmission must be limited, not only for hosts with diarrhoea, but also for those without.

In summary, risk factors for *Cryptosporidium* infection in developing areas vary from study to study (Table 4.19) and variations may be attributed to behavioural and socio-economic differences in various populations, in distinct geographic locations and cultural settings. However, methods of study design and statistical analysis are also likely to account for some of the variation observed and this must be taken into consideration when comparing studies associated with risk factor analysis.

Table 4.19 Comparison of risk factor analysis studies for *Cryptosporidium* from developing countries

Study Design	Statistical analysis	Diagnosis*	Location	Age	Sample size (infected)	Univariate risk factors	Multivariate risk factors	Reference
Cross-sectional	GLM	Diarrhoeal status unknown Formol-ether concentrain, AP stain	Osun state, Nigeria	19.5-72 months	692 (134)	N/A	Overall: *Ascaris* infection, malaria infection, exclusive breastfeeding *C. hominis*: Stunting, younger age, low maternal education *C. parvum*: Stunting, malaria infection	See current thesis
Cross-sectional	Univariate & multivariate logistic regression analysis	Unknown ELISA	Texas-Mexico border (Clinics)	6 mo – 13yrs	285 (196)	Living in urban community Less secondary maternal education School attendance Increased size of household Exposure to farm animals Not breast-fed	Urban living Older age Consumption of municipal water Lower annual household income	Leach *et al.* (2000)

Table 4.19 Contd

Study Design	Statistical analysis	Diagnosis*	Location	Age group	Sample size (infected)	Univariate risk factors	Multivariate risk factors	Reference
Cross-sectional	Univariate analysis	All with acute diarrhoea Sugar flotation, acid-fast kinyoun stain	Mexico (Hospital)	< 5 yrs	403 (26)	Malnutrition Not breast-fed	N/A	Javier-Enriquez *et al.* (1997)
Cross-sectional	Univariate & multivariate logistic regression analysis	All diarrhoeic ELISA	Egypt (Hospital)	< 5 yrs	1275 (214)	Younger age Not breast-fed	Younger age Not breast-fed	Abel-Messiah *et al.* (2005)
Cross-sectional	Multivariate logistic regression analysis	All diarrhoeic Sugar flotation & modified Kinyouns acid-fast stain	Indonesia (Hospital & Community)	Adults & children	4368 (49)	N/A	<2 yrs of age Contact with cats, flood & crowded living conditions	Katsumata *et al.* (1998)
Cross-sectional	Forward stepwise logistic regression analysis	All diarhoeic Direct immuno-fluorescent assay	Brazil (Hospital)	2 wks-10yrs	445 (64)	N/A	Younger age Male Number of children in daycare Children in the household with diarrhoea Dwelling distance from water body (further more risk)	Periera *et al.* (2002)

Table 4.19 Contd

Study Design	Statistical analysis	Diagnosis*	Location	Age	Sample size (infected)	Univariate risk factors	Multivariate risk factors	Reference
Cross-sectional	Univariate analysis	All diarrhoeic Direct smear and mNZ stain	Zambia (hospital)	<11 yrs	222 (39)	Living in areas with high-risk piped water Own their own house Not breast-fed	N/A	Nchito *et al.* (1998)
Case-control	McNemer's test Paired t-test Wilcoxin signed rank test	All diarrhoeic ELISA	India (Hospital)	<5 yrs	46 cases (46 age-matched controls)	< 2 yrs	N/A	Khan *et al.* (2004)
Case-control	Univariate & multivariate logistic regression analysis	All diarrhoeic FE concentration Kinyouns acid-fast stain	India (Hospital)	0-59 mo	68 cases (204 controls)	< 2 yrs Not breast-fed Stunting	< 2 yrs Stunting Not breastfed	Bhattachary a *et al.* (1997)

Table 4.19 Contd

Study Design	Statistical analysis	Diagnosis*	Location	Age	Sample size (infected)	Univariate risk factors	Multivariate risk factors	Reference
Case control	Conditional logistic regression analysis	All diarrhoeic Sugar flotation & modified Kinyouns acid-fast stain	Guinea-Bissau (Community)	< 5 yrs	125 cases 125 controls	N/A	Keeping pigs and dogs in the household Storage of cooked food for later consumption Male child Not breast-fed	Molbak *et al.* (1994)
Cohort study	Cox regression analysis	All diarrhoeic FE concentration & AP stain	NE Brazil (Community)	0 – 4 yrs (followed from birth)	189 (58)	N/A	Low birth weight Living in densely crowded areas	Newman *et al.* (1999)

*Diagnosis consists of diarrhoeal status and methods of detection

4.4.2 Risk factors for *Cryptosporidium* infection in Nigerian children

In the current study, when all *Cryptosporidium* infections were considered for statistical analysis, a number of risk factors emerged. Children infected with *Ascaris* and those that had been breast-fed exclusively (trend observed) had a decreased risk of *Cryptosporidium* infection, while children infected with malaria showed a trend for an increased risk of infection. In addition, a significant interaction was observed between household crowding and the presence of an individual in the household with diarrhoea.

Previous research has shown that patients can be co-infected with *Cryptosporidium* and microparasites such as *Blastocystis hominis*, *Giardia lambia*, *Giardia duodenalis*, *Giardia intestinalis*, *Entamoeba histolytica*, *Cyclospora cayetanensis,* in addition to macroparasites including *Ascaris lumbricoides*, *Trichuris trichiura*, *Strongyloides stercoralis* and *Dicrocoelium dendriticum* (Egloff *et al.*, 2001; Nwabuisi, 2001; Helmy *et al.*, 2006; Kurniawan *et al.*, 2009; Mukherjee *et al.*, 2009). However, the majority of these studies simply detected the presence of these species through the analysis of stool samples, with few determining the physiological and immunological significance of such co-occurrences (Bednarska *et al.*, 2008). Interactions between *Cryptosporidium* and malaria and *Cryptosporidium* and *Ascaris* have not yet been specifically investigated.

In our study infection with *Ascaris* was negatively associated with *Cryptosporidium* infection, such that children infected with *Ascaris* had a decreased risk of becoming infected with *Cryptosporidium.* Although no studies have been conducted to date which focus on co-infections with *Ascaris* and *Cryptosporidium*, infection with *Heligmosomoides bakeri* and *Cryptosporidium* have been explored in the mouse model (Bednarska *et al.*, 2008) with concurrent *H. bakeri* infection resulting in a prolonged course of *C. parvum* infection. In addition, mice infected with *C. parvum* alone exhibited an increased production of IFN-gamma in comparison to control mice, while co-infection with *H. bakeri* resulted in the inhibition of IFN-gamma secretion (Bednarska *et al.*, 2008). Therefore, as IFN-gamma plays a dominant role in the control of *Cryptosporidium* [and this is consistent with previous research (Chen *et al.*, 2002; Riggs, 2002; Pantenburg *et al.*, 2008)], co-infection with *Ascaris* may increase the severity of *Cryptosporidium* infection. However, this positive association was not identified in the present study. A dominance of low intensity *Ascaris* infections in

these Nigerian children (Kirwan *et al.*, 2009) may account for this difference as the intensity of helminth infection is known to influence the strength of the host's immune response (Palmer *et al.*, 1995). However, the intensity of *Ascaris* infection was not taken into consideration in the current analysis and so more detailed investigations are required to determine the physiological and immunological significance of the association between *Ascaris* and *Cryptosporidium*.

Unlike the association between *Cryptosporidium* and *Ascaris*, a positive relationship between *Cryptosporidium* and malaria was detected. From an immunological point of view, interferon (IFN) - gamma, released by a host in response to infection, is considered crucial in host resistance to both malaria (Torre *et al.*, 2001; Graham, 2008) and *Cryptosporidium* (Chen *et al.*, 2002; Riggs, 2002; Pantenburg *et al.*, 2008) infections, indicating that if a child was infected with malaria there could be a decreased risk of infection with *Cryptosporidium* (owing to the production of high levels of IFN-gamma during malaria infection). However, the opposite relationship was observed in our study. Host susceptibility to protozoan infections could explain the positive association. As discussed by Faulkner *et al.* (2005) (in relation to concomitant helminth infections), genetic regulation may occur through non-immune mediated processes that determine susceptibility to infection as well as through variation in capacity to mount a protective response.

It is important to note that data on malaria infection were obtained from malaria rapid test results. The intensity of malaria infection will be confirmed by parasitemia counts of prepared blood smears in order to verify the association between *Cryptosporidium* and malaria and further work on co-infections with *Cryptosporidium* is required before the relationships between *Cryptosporidium* and malaria, and *Cryptosporidium* and *Ascaris* can be confirmed.

A trend towards the protective effect of exclusive breast-feeding for the first 6 months of life was observed in the present study and results are consistent with studies from India, Guinea Bissau, Egypt, Mexico and Tanzania which have linked the protective nature of breast-feeding to a reduced risk for *Cryptosporidium* infection (Molbak *et al.*, 1994; Bhattacharya *et al.*, 1997; Javier Enriquez *et al.*, 1997; Nchito *et al.*, 1998; Abdel-Messih *et al.*, 2005). This reduced risk may be as a result of the protective effect of breast-feeding, or from limited contact with potentially infective food and water, or surfaces prior to walking (Nichols, 2008). In contrast, studies from

Brazil, India and Mexico have found no association between breast-feeding and *Cryptosporidium* infection (Newman *et al.*, 1999; Leach *et al.*, 2000; Pereira *et al.*, 2002; Khan *et al.*, 2004).

Although neither the presence of a member in the household with diarrhoea or household crowding were independent risk factors for infection in the current study, an interaction between the 2 variables was observed. This indicated that although the presence of an individual in the household suffering from diarrhoea (which may be an indication of cryptosporidiosis) is not associated with risk in houses with few individuals, their presence becomes important in crowded households (possibly owing to increased person-to-person contact) and leads to an increased risk of infection. Direct person-to-person transmission has been observed previously in day-care centres, nurseries, within hospitals and also within families (Koch *et al.*, 1985; Goodgame *et al.*, 1993; Newman *et al.*, 1994; Fayer *et al.*, 2000; Pandak *et al.*, 2006; Turabelidze *et al.*, 2007). Person-to-person contact within families was indicated as a risk factor for infection in Bangladesh, with 12.5% of family members contracting cryptosporidiosis 3 weeks following the diagnosis of infection of a child in the household (Rahman *et al.*, 1990). In addition, in Mexico, an outbreak of cryptosporidiosis occurred in a paediatric hospital. The source of infection was traced to a single infant and hospital staff were implicated as the most likely cause of disease spread. When assessed, it was discovered that only 30% of staff were washing their hands before attending to an infant (Navarrete *et al.*, 1991).

4.4.3 Risk factors by species

Recent data have indicated that clinical manifestations for *Cryptosporidium* vary among species and subtypes (Bushen *et al.*, 2007; Cama *et al.*, 2007; Cama *et al.*, 2008) and there is evidence to suggest that distinct sources and, therefore, transmission routes exist for *C. hominis* and *C. parvum* due to variations observed in host ranges (with *C. hominis* limited primarily to human infections and *C. parvum* linked to both zoonotic and anthroponotic infection). This suggests that sufficient variation exists between species and subtypes such that risk factors for infection may also differ. To date, only 5 studies, 2 from the developed world (USA and Australia) and 3 from developing regions (Peru and India) have assessed risk factors separately by species and in the case of the 2 studies from Peru, by subtypes (Hunter *et al.*,

2004; Ajjampur *et al.*, 2007; Cama *et al.*, 2007; Lake *et al.*, 2007; Cama *et al.*, 2008). The study design used for each of these studies is presented in Table 4.20. Case-control (2 studies), cohort (2 studies) and cross-sectional (1 study) designs were adopted, and the diarrhoeal status, age group, sample size and methods for statistical analysis varied among the studies.

In England and Wales, a case-control study was conducted using a logistic regression model, to identify risk factors for sporadic cryptosporidiosis in adults and children (Hunter *et al.*, 2004). The overall model, incorporating 115 *C. hominis* and 76 *C. parvum* isolates, identified travel outside the UK, contact with persons with diarrhoea, touching cattle, toileting contact with a child less that 5 years of age and number of glasses of unboiled water consumed as significant risk factors for infection, while eating ice cream, raw vegetables and tomatoes were considered protective. However, when analysed separately for *C. hominis* and *C. parvum,* risk factors varied by species. For *C. hominis*, travel abroad and changing diapers were associated with infection while touching farm animals was associated with *C. parvum* infection only (Hunter *et al.*, 2004).

Similarly, in a subsequent study, also carried out in England and Wales, a case-control analysis was conducted to investigate the role of wider environmental and socio-economic factors upon human cryptosporidiosis (Lake *et al.*, 2007) and *C. parvum* and *C. hominis* were examined separately. Questionnaires were not used for the collection of information in this study. Instead, GIS mapping was used to obtain information about the living areas of laboratory-confirmed cryptosporidiosis cases. Results from the overall model and the *C. parvum* model identified a number of risk factors, namely, living in areas with (1) many higher socio-economic status individuals (2) many children aged less than 4 years, (3) a high estimate of *Cryptosporidium* applied to the land from manure and (4) living in areas with poorer water treatment. However, for *C. hominis*, differing risk factors were identified, namely, living in (1) urban areas (2) areas with a large number of children aged less than 4 years and (3) living in areas with many people of higher socio-economic status. Although collecting data from questionnaires can introduce recall bias, information collected by GIS mapping may not represent a true picture of the population being tested as, within a geographical location, no matter how small, it is likely that not all individuals will behave in the same way and so caution must be taken when considering the results of the above mentioned study.

As risk factors will vary among regions and populations, studies from developed countries may not represent the best comparison of risk factors for our study. However, both studies described above do indicate that risk factors can vary by species and suggest that future research should focus on this method of analysis. Studies from developing countries are required to better understand risk factors by species in tropical areas and to date, just 3 studies have been carried out in developing regions (Ajjampur *et al.*, 2007; Cama *et al.*, 2007; Cama *et al.*, 2008) (see Table 4.20).

In Peru, the analysis of risk factors by species and subtypes (*C. hominis* Ia, Ib, Id, Ie, *C. parvum* IIc, *C. meleagridis* and *C. canis* and *C. felis* pooled) indicated that HIV-infected individuals who had been in contact with children <5 years of age were more likely to be infected with *C. hominis* subtype family Ie and, as discussed by the authors, this finding is in concordance with the anthroponotic nature of *C. hominis* (Cama *et al.*, 2007). However, no other risk factors (person-to-person contact, animal contact, food and waterborne variables and sexual practices) were identified in this Peruvian study. A second study conducted in Peru assessed risk factors for *Cryptosporidium* infection in children but did not identify any risk factors for infection between any *Cryptosporidium* species or subtype families (*C. hominis* Ia, Ib, Id, Ie, *C. parvum* IIc, *C. meleagridis* and *C. canis* and *C. felis* (pooled) were compared) even though the analysis included aspects of sanitation and zoonotic, foodborne and waterborne transmission (Cama *et al.*, 2008). The authors proposed that the lack of an identification of risk factors was due the occurrence of mostly anthroponotic subtypes (*C. hominis* and *C. parvum* IIc) and that children in the study were being constantly exposed to the ubiquitous parasite through various transmission routes (Cama *et al.*, 2008).

A third study from India investigated risk factors between *C. hominis*-infected children (with diarrhoea) and other species (*C. parvum*, *C. felis* and *C. parvum* mouse genotype) but found no significant differences in demographic (age, gender, birth order, educational status of the mother or occupation of the father) or clinical characteristics between *C. hominis*-infected children and those infected with other species using chi-squared analysis (Ajjampur *et al.*, 2007).

The above mentioned studies present contrasting results with some suggesting that risk factors vary by species (Hunter *et al.*, 2004; Cama *et al.*, 2007; Lake *et al.*, 2007), while others have failed to identify risk factors between species or subtypes, suggesting a lack of variation between risk factors (Ajjampur *et al.*, 2007; Cama *et al.*, 2008).

In the current study, a total of 134 positive cases (558 negative) were included for analysis in the full model. Species were identified in 49 cases and so for the remaining positives, species identity was not determined. As some studies have indicated that risk factors vary by species (Hunter *et al.*, 2004; Cama *et al.*, 2007; Lake *et al.*, 2007), a model was created to assess risk factors for *C. hominis* and *C. parvum* together and for each species separately. Although the power of a study is reduced when the analysis is restricted to cases where the species have been identified, when species are grouped, the analysis may highlight factors common to both species, but may downplay risk factors associated with one species or the other. Therefore, the current study determined the species of *Cryptosporidium* causing the infections and investigated *C. hominis* and *C. parvum* both together and separately for risk factor analysis. Even with reduced power the GLM analysis identified a number of risk factors for infection. The major impact of low n-values on the analysis was that interactions between risk factors could not be determined by species. Initially, interactions were investigated by species but risk factors which were highlighted were found to be artefacts of low sample size and therefore were not further investigated.

For children infected with either *C. hominis* or *C. parvum*, risk of infection decreased with increasing age and with an increasing level of maternal education. Conversely, the risk of infection increased with malaria infection, and with increasing severity of stunting.

Younger age was associated only with *C. hominis* infection suggesting that younger children are at higher risk of infection if *C. hominis* is circulating in the population rather than *C. parvum*. This is in contrast to findings in southern India which found no significant differences in age between *C. hominis*-infected children and those infected with other species (Ajjampur et al., 2007). However, *C. parvum* was grouped with *C. felis* and *C. parvum* mouse genotype and so results are not entirely comparable with the present study.

Although the majority of studies have not determined risk factors by species, younger age is well documented as a risk factor for *Cryptosporidium* infection with studies from Egypt, Nigeria, Gambia, Malawi, Bangladesh and Indonesia indicating that children < 2 years are most susceptible (Adegbola *et al.*, 1994; Katsumata *et al.*, 1998; Nwabuisi, 2001; Khan *et al.*, 2004; Abdel-Messih *et al.*, 2005; Morse *et al.*, 2007). In Egypt, children < 1 year old were 2.4 times more likely to be infected than older children (up to 5 years) and children aged 12-23 months were 1.9 times more likely to be infected than 24-36 month old children (Abdel-Messih *et al.*, 2005). In contrast, on the Texas-Mexico border, children aged 6 months to 13 years were assessed and it was found that older age was associated with infection (Leach *et al.*, 2000). Leach *et al.* (2000), suggest that a higher risk of infection in older age groups may be due to increased cumulative risk with advanced age. However, contrasting studies assessing both children and adults from England, Wales, Mexico, Kuwait, Bolivia and India have found no link between age and risk of infection (Ajjampur et al., 2007; Das et al., 1993; Esteban et al., 1998; Sulaiman et al., 2005; goh 04; Hunter 04; Javier 97). It is possible that contrasting results have emerged due to regional population differences or differences in host behaviour within age groups in different regions or, as previously discussed, may be a result of variations in study design.

A relationship was observed between stunting (height-for-age) and both *C. parvum* and *C. hominis* infection, with increasing severity of stunting leading to a greater risk of *Cryptosporidium* infection. Causation was not identified in the current study and so we can not determine whether stunting is caused by the infection or whether children are predisposed to infection when stunted. Previous studies from Mexico, India and Peru have also identified a similar relationship between *Cryptosporidium* infection and stunting (Bhattacharya *et al.*, 1997; Checkley *et al.*, 1997; Javier Enriquez *et al.*, 1997; Checkley *et al.*, 1998).

A longitudinal study was conducted over a 2-year period in Peru to explore the effects of *Cryptosporidium* infection on child growth during the year following the onset of first infection. Children aged 0-3 months were recruited and monitored twice weekly for diarrhoeal status, once weekly for stool examination and once a month for height and weight measurements (Checkley *et al.*, 1998). A lasting adverse effect on linear growth (stunting) was identified, in both symptomatic and asymptomatic children,

with a greater effect observed in younger children (0-5 months) and those stunted prior to infection. These results are consistent with results from Uganda where the authors conducted a cross-sectional, case-control study on children aged 0-60 months in order to determine risk factors for persistent diarrhoea (Tumwine *et al.*, 2003). Taking the mean height-for-age, weight-for-age and weight-for-height z-scores, chi-squared analyses identified a significant association between *Cryptosporidium* infection and stunting, being underweight and wasting.

The species of *Cryptosporidium* which infected the children in the above mentioned studies on malnutrition were not determined. However, in Brazil, a birth cohort was assessed over a 5-year period to determine the relationship between *Cryptosporidium* species and malnutrition (Bushen *et al.*, 2007). *C. hominis* was identified in 42 infections and *C. parvum* in 18. While weight-for-age (underweight) was not associated with any infections, stunting decreased significantly within 3 months of infection for children harbouring either *C. hominis* or *C. parvum*. However, 3-6 months following infection, only *C. hominis*-infected children continued to demonstrate stunting indicating increased pathogenicity for *C. hominis* infections (Bushen *et al.*, 2007).

Ascaris infection was highlighted as a risk factor for all species of *Cryptosporidium* but not for *C. hominis* and *C. parvum* separately. This may indicate that there is no association between *Ascaris* and the 2 *Cryptosporidium* species or simply that the relationship was not strong enough to detect when the sample size decreased to identify risk factors separately by species. Therefore, additional studies (with larger sample sizes) are required in order to investigate this relationship between *Ascaris* and *Cryptosporidium*.

In the present analysis, children infected with malaria were more likely to be infected with *C. parvum* rather than *C. hominis*. The significance of this relationship is not yet clear and, as discussed previously, data on malaria intensity will be required in order to confirm the association.

Prior to the construction of the generalised linear model (GLM) a number of variables, namely, level of maternal education, socio-economic index, hygiene index and animal contact frequency index were strongly correlated. As GLMs are not built to deal with correlated variables, each variable was analysed independently and the best fitting

model was retained. The results of the GLM analysis indicated that the level of mother's education provided the best fit model. Therefore, this variable was included in the final model and the correlated variables were dropped. However, it is possible that each of the correlated variables are also risk factors for *Cryptosporidium* infection.

A lack of maternal education was a significant risk factor for infection with *C. hominis* or *C. parvum,* and for *C. hominis* infections alone. Few studies have identified parental education as a risk factor for *Cryptosporidium*. Leach *et al.* (2000) found an association between mothers having less secondary education and an increased risk of *Cryptosporidium* infection but this did not remain significant in their logistic regression model. Parents with a higher level of education may exhibit behaviours (e.g. hygiene awareness) that would reduce exposure to infection in children. For example, when the hygiene index from the current study was divided into low, medium and high there was a statistically significant association between the level of maternal education and hygiene class, with 37.9% of mothers with no education in the low hygiene group and 20.7% in the high hygiene group, compared to 16.5% of mothers with a tertiary level education in the low hygiene group and 31.8% in the high hygiene group. Likewise, 58.6% of mothers with no education did not wash their hands after using the toilet compared with 26.4% of mothers with tertiary level education.
Socio-economic status has not been previously evaluated as a risk factor for *Cryptosporidium* infection, however, in Bangladesh, household income was assessed but was not identified as a risk factor for infection in children less than 5 years of age with diarrhoea (Bhattacharya *et al.*, 1997).

Statistical associations between *Cryptosporidium* infection and animal contact have been observed on a number of occasions. In Australia, touching cattle was a risk factor for all *Cryptosporidium* infections but touching farm animals was only a risk factor for *C. parvum* infection (Hunter *et al.*, 2004). In addition, in Indonesia, cat ownership was identified as a risk factor (Katsumata *et al.*, 1998), while pigs and dogs present in the household were implicated as risk factors in Guinea Bissau (Molbak *et al.*, 1994). In contrast, exposure to pets was considered protective in Melbourne (Robertson *et al.*, 2002), while no association between animal exposure and infection was identified in many other studies (Chacin-Bonilla *et al.*, 1993; Javier Enriquez *et al.*, 1997; Nchito *et al.*, 1998; Newman *et al.*, 1999; Pereira *et al.*, 2002;

Goh *et al.*, 2004; Khan *et al.*, 2004; Roy *et al.*, 2004). In all Nigerian villages assessed in the present study, children were observed living in close proximity to animals such as goats, dogs and chickens which roamed freely among the houses. Thus, the transmission of *Cryptosporidium* species to humans was potentially high via direct contact with animals or faecal matter or indirectly through contact with contaminated food, water or surfaces. However, the infection status of these animals was undetermined as faecal samples were not analysed.

Poor personal hygiene practices have been long established as a route of faecal – oral transmission of diarrhoeal diseases (Ferrer *et al.*, 2008) and have been reported as a possible cause of cryptosporidiosis outbreaks in developed countries associated with hospitals and day care centres (Walters *et al.*, 1988; Navarrete *et al.*, 1991).

Although results from our risk factor analysis clearly indicate that distinct differences are present between *C. hominis* and *C. parvum*, the majority of risk factors that emerged from the analysis were not necessarily associated with transmission routes for infection, but rather with the health or immune status of the child (stunting, malaria infection, *Ascaris* infection and age). Therefore, it is possible that sources and transmission routes for infection in this population were similar for both species. This result may not be surprising as at least 68% of the *C. parvum* isolates were of the anthroponotic subtype IIc (see Chapter 3, Section 3.3).

Table 4.20 Risk factors by species from developed and developing countries

Study Design	Statistical analysis	Diagnosis*	Location	Age	Sample size (infected)	Univariate risk factors	Multivariate risk factors	Reference
Cross-sectional	GLM	Diarrhoeal status unknown Formol-ether concentrain, AP stain	Osun state, Nigeria	19.5-72 months	692 (134)	N/A	Overall: *Ascaris* infection, malaria infection, exclusive breastfeeding *C. hominis*: Stunting, younger age, low maternal education *C. parvum*: Stunting, malaria infection	See current thesis
Cross-sectional	Multivariate	Diarrhoeic & non-diarrhoeic Modified Ritchie formalin ether concentration & modified acid-fast stain	Peru (Hospital)	>17 yrs (HIV +)	2490 (230)	N/A	Contact with children <5 yrs for *C. hominis* Ie	Cama *et al.* (2007)
Case-control	Univariate & Multivariate	Cases all with diarrhoea mZN & AP stain	England & Wales	Adults & children	427 cases 427 controls	See http://www.cdc.gov/ncidod/eid/vol10no7/03-0582.htm#app	See Appendices Table A1.1	Hunter *et al.* (2004)
Case-control	Multivariate	Cases all with diarrhoea Unknown	England & Wales	Adults & children	3343 cases 3343 location-matched controls	N/A	See Appendices Table A1.1	Lake *et al.* (2007)

Table 4.20 Contd

Study Design	Statistical analysis	Diagnosis*	Location	Age	Sample size (infected)	Univariate risk factors	Multivariate risk factors	Reference
Cohort	Univariate	All diarrhoeic Acid-fast stain	India (Community)	0-3 yrs (followed from birth)	1949 (58)	No risk factors identified between *C. hominis* infection & other species	N/A	Ajjampur *et al.* (2007)
Cohort	Univariate	Diarrhoeic & non-diarrhoeic Modified Ritchie formalin ether concentration & modified acid-fast stain	Peru (Community)	0-3 yrs (followed from birth)	533 (109)	No risk factors identified between species and subtypes	N/A	Cama *et al.* (2008)

*Diagnosis consists of diarrhoeal status of cases and methods of detection

4.4.4 Non-significant variables

A number of risk factors which were included in the statistical models remained non-significant. Our study found no difference for risk of infection between males and females for all infections or those divided by species. This finding is analogous to research from developed countries such as England and the US (Goh *et al.*, 2004; Roy *et al.*, 2004) and developing countries including Malawi, Indonesia, Bolivia, Gambia, Mexico, Jordan, Kenya, Brazil, India, Zambia and Egypt (Adegbola *et al.*, 1994; Miller *et al.*, 1994; Nimri and Hijazi, 1994; Bhattacharya *et al.*, 1997; Esteban *et al.*, 1998; Katsumata *et al.*, 1998; Nchito *et al.*, 1998; Newman *et al.*, 1999; Khan *et al.*, 2004; Abdel-Messih *et al.*, 2005; Gatei *et al.*, 2006a; Morse *et al.*, 2007) where gender was not found to be associated with risk of infection. In addition, Ajjampur *et al.* (2007) determined the species of *Cryptosporidium* present in a paediatric population and found no association with gender of the child between *C. hominis*-infected children and those infected with other species. In contrast, 2 studies from Nigeria indicated that female children had a higher prevalence of *Cryptosporidium* infection than males (Kwaga *et al.*, 1988; Okafor and Okunji, 1994) consistent with findings from Guatemala and Guinea-Bissau (Molbak *et al.*, 1994; Laubach *et al.*, 2004). Conversely, male children in Brazil were found to be 2.2 times more likely to be infected than females (Pereira *et al.*, 2002).

Risk factor analysis from Australia and the US (Robertson *et al.*, 2002; Hunter *et al.*, 2004) highlighted contact with persons with diarrhoea and toileting a child under 5 years as risk factors for infection. Similarly, the number of children in the household with diarrhoea was associated with risk of infection in Brazil (Pereira *et al.*, 2002). However, living with a person with diarrhoea was not associated with risk of *Cryptosporidium* infection in the current study, although as previously discussed, a significant interaction between living with an individual with diarrhoea and household crowding was observed.

Failure to boil water before drinking was not associated with *Cryptosporidium* infection in the current study. Previous research has indicated that unboiled rural water in Australia (Robertson *et al.*, 2002), the number of glasses of unboiled tap water consumed in England and Wales (Hunter *et al.*, 2004), unboiled tap water in N. Cumbria, England (Goh *et al.*, 2004) and high-risk piped water in Zambia (Nchito *et al.*, 1998) were associated with infection. Infection through the contamination of water has been long established as a transmission route for the parasite. Most notably, with respect to the Milwaukee outbreak in 1993, when

untreated water from Lake Michigan entered the public water supply and resulted in a large-scale cryptosporidiosis outbreak affecting over 400,000 people (Mackenzie *et al.*, 1994). However, studies conducted in Indonesia and Mexico have found no association between drinking untreated water and infection in adults and children with diarrhoea (Katsumata *et al.*, 1998) nor the use of unboiled water and infection in children under 5 years with acute diarrhoea (Javier Enriquez *et al.*, 1997).

4.4.5 Summary

The identification of risk factors for *Cryptosporidium* infection is vital in order to elucidate the transmission dynamics of the parasite. To date, no effective therapy has been developed for the treatment of *Cryptosporidium* infection, therefore exposure to infectious oocysts must be reduced through the implementation of appropriate and cost effective control programs.

A number of risk factors for sporadic *Cryptosporidium* infection in Nigerian children were identified in our study and although risk factors were identified which varied by species (*C. hominis*: Stunting, younger age and low maternal education; *C. parvum*: Stunting and malaria infection), differences seemed to be related to the health status of the child rather than to behavioural differences.

Thus, additional studies are required in order to obtain further evidence for a variation in risk factors between species and / or subtypes, in various regions and populations, in both developed and developing countries.

5 General Discussion

Section **Page**

5.1 General Discussion

Cryptosporidium has become increasingly recognised as an important human pathogen and in 2004 was included in the WHO's 'Neglected Diseases Initiative'. Infections included in this initiative occur in 'developing countries where climate, poverty and lack of access to services influence outcomes' and which 'exhibit a considerable and increasing global burden, and impair the ability of those infected to achieve their full potential, both developmentally and socio-economically, in missed opportunities' (Savioli *et al.*, 2006). As further insight into the epidemiology of *Cryptosporidium* and the implications for child health is required, especially in developing countries, the overall aim of this study was to elucidate the epidemiology of *Cryptosporidium* in a high-risk paediatric population. In order to accomplish this, the prevalence and temporal variability of infection was established in Nigerian children and the species of *Cryptosporidium* causing the infections were identified by molecular analysis. Furthermore, risk factors for infection were determined by means of a cross-sectional study and robust statistical analysis. To our knowledge this is the first holistic study on *Cryptosporidium* epidemiology to be conducted in West Africa.

Following analysis of a large, representative sample of Nigerian children, the prevalence of *Cryptosporidium* infection was found to range from 15.6% to 19.6%, considerably higher than prevalence rates determined in children from other developing countries (Adegbola *et al.*, 1994; Javier Enriquez *et al.*, 1997; Katsumata *et al.*, 1998; Iqbal *et al.*, 1999; Iqbal *et al.*, 2001; Pereira *et al.*, 2002; Khan *et al.*, 2004; Abdel-Messih *et al.*, 2005; Cordova Paz Soldon *et al.*, 2006; Ajjampur *et al.*, 2007). Higher infection rates are generally associated with high-risk groups such as young children and those with HIV/AIDS. Although the immune status of the children was not determined in our study, it is unlikely that HIV had a great influence on the prevalence of *Cryptosoridium* infection in this population, as the overall rate of HIV infection in Nigeria is low relative to other African nations (3.1% in 15-49 year olds, WHO 2006). Prevalence of infection varied significantly over time in this region of Nigeria, indicating that during May and February 2007, there was a greater risk of infection in comparison with September 2006. Rainfall did not appear to contribute to the temporal variation and further insight into behaviours and practices within the community, which may vary over time, is required before conclusions may be drawn regarding the driving force behind the observed seasonal variation.

Although the most up-to-date microscopy methods were adopted for the detection of oocysts in faecal samples (including concentration and staining techniques), the sensitivity of detection must be evaluated. A number of factors are likely to contribute to a decreased sensitivity of detection when assessing the prevalence of *Cryptosporidium* in stool samples. Firstly, the examination of an individual pea-sized amount of faeces, from a single stool, is not likely to be sufficient in detecting all cases of infection. Preferably, multiple stool samples would be taken from a child, over a short period of time, and replicate slides for each sample would be created for microscopy. Secondly, there is often a considerable amount of debris in stool samples, even after concentration techniques have been performed, and this debris may mask the presence of oocysts on the slide. In the present study the masking effect may have been amplified owing to the presence of very low intensities of oocysts and as discussed by Smith (2008), low oocyst excretors (e.g. asymptomatic hosts) will 'probably not be diagnosed, because oocyst numbers will be below the limit of detection of the conventional methods utilised'. In addition, Periera *et al.* (2002) states that there is a 5% likelihood of generating false negatives when assessing *Cryptosporidium* infection and so it is likely that in the current study, some children were misclassified as uninfected. This indicates that there may be an underestimation of the prevalence of infection in our study.

Previous research has shown that a range of *Cryptosporidium* species, genotypes and subtypes infect humans and each may have different sources of infection, transmission routes, pathogenicity and even contrasting risk factors for infection. Therefore, in order to limit exposure to infectious oocysts, identifying the species present in a population is crucial. Results from the current study found a high diversity of species, genotypes and subtypes infecting these Nigerian children, including *C. hominis*, *C. parvum*, mixed infections of *C. hominis* and *C. parvum*, *C. canis, Cryptosporidium* cervine genotype and *Cryptosporidium* rabbit genotype. This study is the first study to isolate the cervine genotype from humans in Africa and the first, outside the UK, to isolate the rabbit genotype from humans (Robinson *et al.*, 2008b; Chalmers *et al.*, 2009a). In addition, *C. canis* was isolated on only 1 occasion previously in humans in Africa (Gatei *et al.*, 2006a). A range of GP60 subtypes of *C. hominis* and *C. parvum* were also isolated and included a novel subtype (Ih) of *C. hominis* and 2 subtypes (IIi and IIm) of *C. parvum* both of which have been identified in humans on only 1 occasion previously (Akiyoshi *et al.*, 2006). Furthermore, high levels of the anthroponotic *C. parvum* IIc subtype were isolated. These results have implications for

unravelling the epidemiology of *Cryptosporidium* as the data suggest that (1) children are a source of infection for a range of species, genotypes and subtypes in the community and (2) that a high level of *C. hominis* infection, coupled with the dominance of the *C. parvum* subtype IIc indicates that anthroponotic rather than zoonotic transmission is prevalent in the area. Therefore, when implementing control programmes, it appears that emphasis should be placed on the importance of personal hygiene and better sanitation [as current data indicated that few children use flush toilets or pit latrines (4%) and seldom wash their hands after defecation (37.1%)] in order to limit anthroponotic transmission, rather than focussing attention on reducing zoonotic transmission.

It is interesting to note that a number of species and subtypes appear to have a pan-global distribution. It is likely that the rise in global sourcing and rapid transport of food and, increased human travel has allowed for the transmission of the parasite around the world. In addition, the fact that *Cryptosporidium* does not require a specific intermediary host for completion of the life cycle and that it can survive under a range of environmental conditions indicates that the parasite is likely to survive in a range of geographical locations including both temperate and tropical zones. However, it may be somewhat premature to make strong statements on the global distribution of particular subtypes and the apparent limited distribution of others as the picture is far from complete. The current trend may simply reflect the work that has been conducted to date and thus may introduce an element of bias. Therefore, additional studies are required to investigate the distribution patterns of various species and subtypes in a range of geographical locations.

Furthermore, subtypes of *C. meleagridis* were identified and as subtyping results provide us with a more discriminatory system for identifying sources of infection and routes of transmission in both *C. hominis* and *C. parvum,* it is probable that subtyping of *C. meleagridis* will provide the same enhanced understanding. The current study identified *C. meleagridis* Type 1 in all 3 samples analysed. As no community-based study has investigated *C. meleagridis* subtyping, the importance of the finding remains unclear. Further epidemiological studies are required before the significance of *C. meleagridis* subtyping can be determined.

The molecular techniques applied to amplify oocyst DNA for species identification represent some of most advanced methods available. Water-ether concentration was performed to increase oocyst abundance and separate the oocysts from particulate matter, thereby reducing PCR interferents. Concentration was followed

by mechanical disruption (freeze-thawing) to disrupt the cell membranes and release DNA. In addition, PVPP and Chelex were added to the DNA to bind to heavy metals which act as catalysts for the degradation of DNA at high temperatures in low ionic strength solutions (Smith and Nichols, 2009b) and to remove polyphenolics and polysaccharides, which can deactivate proteins and inhibit PCR reactions, possibly through an adverse effect on Taq polymerase (Smith and Nichols, 2009b). Nested PCR assays were used as they increase detection sensitivity, especially if oocyst abundances are low and 2 loci at the SSU rRNA gene were targeted in order to further increase sensitivity for detection for a range of *Cryptosporidium* species. Furthermore, samples with an ambiguous RFLP pattern were sequenced to confirm the true species identity.

In spite of the precautions taken to ensure increased amplification of oocysts, species were identified in a low percentage of samples. This was possibly owing to the low intensity of oocysts which were noted during microscopy. However, stool consistency also varied from solid to fluid (with variation possibly occurring owing to disease state, diet or age) and consistency can influence oocyst recovery (Smith, 2008). In addition, faecal samples can contain a number of PCR inhibitors such as bilirubin, bile salts, polyphenolics and polysaccharides and so it is possible that despite the precautions taken to remove inhibitors, some remained and interfered with the PCR reactions. It is also possible that the low numbers of samples that amplified was associated with the long storage time prior to molecular analysis (up to 1 year). However, due to time and cost restraints this was not further investigated. Although optimisation of techniques was attempted during our analysis, these procedures did not significantly increase the amplification of *Cryptosporidium* DNA. It is proposed that the preparation methods for molecular analysis require modification such that a larger amount of the sample may be used in the initial stages or, alternatively, duplicates or triplicates of each sample should be prepared to increase the chances of detecting oocysts. Although the use of IMS did not significantly improve the detection rate of positive samples (over water-ether concentration) in the current analysis, Robinson *et al.* (2008a) recently modified and evaluated the IMS procedure in stools of varying consistency. An increased amount of stool sample, up to 2 g, was used during this procedure (in comparison with the 0.5 g used in the current study) and each sample was pre-treated with Mucolyse (to reduce inhibition from mucus). The analytical sensitivity of the method was found to be <5 oocysts per gram, regardless of stool consistency. The method was determined to be more effective than immunofluorescence but due to small sample sizes comparison with PCR results could not be deemed significant. Although more studies are required to

support the finding, this method may represent an enhanced methodology for the detection of low numbers of oocysts in human faecal samples.

The final aspect of this project involved the investigation of risk factors for *Cryptosporidium* infection in these Nigerian children. As no effective drugs have been developed to treat infected patients, understanding risk factors in order to limit and prevent transmission is essential. Previous research has indicated that risk factors vary by species and even subtypes (Hunter *et al.*, 2004; Cama *et al.*, 2007; Lake *et al.*, 2007), while other studies have found no such variation (Ajjampur *et al.*, 2007; Cama *et al.*, 2008). Thus, our analysis was performed on all *Cryptosporidium* infections, followed by analysis for *C. hominis* and *C. parvum* infections separately. Initially, it was anticipated that risk factors would be detected for all species individually and possibly even for subtypes, however, due to low sample sizes, only risk factors for *C. hominis* and *C. parvum* could be analysed.

Case-control studies have previously been designed to investigate risk factors for sporadic *Cryptosporidium* infection as they represent a quick and low cost method for analysis (Gail and Benichou, 2000). However, the case-control study design may introduce selection bias by choosing particular controls (as controls may not represent the population at risk) (Gail and Benichou, 2000), rather than by random sampling of the population, and so it may not be possible to extrapolate results to the full population. The current investigation therefore adopted a cross-sectional study design and robust statistical analysis was carried out on the data using a binomial generalised linear model.

Multiple chi-squared tests were not carried out on the data prior to the development of the GLM (as previous studies have done) as a primary assumption of the chi-squared test is that it is the only test being carried out on the entire data set. Every time an additional test is conducted on the same group of data, the chance of finding some significant association increases. If multiple comparisons are to be made, the p value for each test should to be adjusted using, for example, the sequential Bonferroni adjustment to correct for multiple comparisons (if *n* dependent or independent hypotheses are being tested on a set of data, then in order to maintain the family-wise error rate, each individual hypothesis must be tested at a statistical significance level of 1/n times p (e.g. p=0.05) (Hochberg and Tamhane, 1987). However, the Bonferroni adjustment is not accepted by all statisticians (Perneger, 1998) and so a robust binomial GLM

analysis (which can also take interactions into account) was adopted for the current investigation.

Our results indicated that risk factors vary for *C. hominis* and *C. parvum*. While stunting increased the risk of infection for both species, low levels of maternal education and younger age were identified as risk factors for *C. hominis*, while malaria infection was a risk factor for *C. parvum* only. However, the risk factors identified (with the exception of the level of maternal education) appear to be associated with the health/immune status of the children, rather than with socio-economic and behavioural variables. Although these results provide novel and important data regarding possible variations in susceptibility to infection among species, information which will enable the implementation of control measures to reduce transmission of infectious oocysts is limited. Although possible sources of infection have been identified in the present study from molecular analysis, risk factors for transmission may be more difficult to identify for a number of reasons. (1) The presence of a high level of anthroponotic species in the population, (2) the existence of many transmission routes which overlap by source and (3) the ubiquitous nature of the parasite. Therefore an understanding of the species, genotypes and subtypes present in an area may represent a greater insight into the sources of infection for *Cryptosporidium*. If high levels of anthroponotic subtypes are identified in an area, then control measures for reducing person-to-person contact are most appropriate. In the current study, the majority of species and subtypes circulating in the population were anthroponotic rather than zoonotic subtypes. Therefore, increased education relating to anthroponotic transmission and improved sanitation conditions should be introduced into the area in an attempt to limit transmission. A small number of zoonotic species were also identified in this population, namely the cervine genotype (3), the rabbit genotype (5) and *C.canis* (1). Although these genotypes and species are associated with zoonotic sources it is possible that the initail source of transmission was linked to an animal but now the main mode of transmission is from human to human. Although rabbits are found in Africa, none were observed in the area over the entire length of the study and mothers did not highlight their presence when indicating the animals they owned or were in contact with. It is possible that rabbits are living unnoticed in the bush and, river water or crops become contaminated with the oocysts excreted. Alternatively, it is possible that the genotype was introduced into the area by humans which have traveled to other locations and returned infected with the genotype. As the rabbit genotype has previously only been recorded in the UK, further epidemiological and molecular analysis is required in various geographic locations in order to determine the

distribution of the rabbit genotype and to determine its pathogenic potential and public health significance.

In conclusion, our study has revealed novel aspects of the epidemiology of *Cryptosporidium* in Nigerian children. A high prevalence of infection and seasonality was noted and a diverse range of both species and subtypes were isolated, including a novel subtype of *C. hominis* (Ih). In addition, this was the first study to isolate the cervine genotype in humans in Africa and the first outside the UK to isolate the rabbit genotype in humans. Humans were implicated as the main source of infection in the population with either direct or indirect anthroponotic transmission likely to be responsible for the spread of the infection.

Future studies on the epidemiology of *Cryptosporidium* should continue to focus on the molecular analysis of the parasite in order to elucidate possible sources of infection in varying regions and populations. In addition, samples from the environment, animal stool samples and household surfaces should also be examined in order to make a direct link between sources of infection, transmission routes and human infection. Furthermore, variations in clinical manifestations between species, genotypes and subtypes should be assessed in conjunction with non-gastrointestinal symptoms such as mental development, to determine which species are most pathogenic and lead to a greater public health risk.

Information regarding the epidemiology of *Cryptosporidium* is lacking, especially in developing regions where the impact of cryptosporidiosis is likely to be significant. Therefore, further epidemiological studies, similar to the current study, should be conducted in various geographical locations, as well as in different groups of individuals, so that the public health significance can be assessed and programs implemented to reduce the spread of infectious oocysts. Crucially, researchers should endeavour to standardise epidemiological methods, in terms of statistical analyses, age groups targeted and methods for detection, so that studies can be more easily compared and results extrapolated to other regions.

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Appendices

Appendix 1

Risk factors for sporadic *Cryptosporidium* infection in developed countries

Table A1.1 Case-control studies from developed countries exploring risk factors for sporadic *Cryptosporidium* infection

Location	Age group	Cases/ Controls	Species data	Risk factors	Protective factors	No Association	Reference
Melbouurne & Adelaide, Australia	Adults & children	335 cases 1331 controls	No	Swimming in Public pools Contact with persons with diarrhoea Calf contact Unboiled rural water Calf contact	Pet exposure Uncooked carrots	Drinking water (tap) Breastfeeding Education level	Robertson *et al.* (2002)
NW England and Wales	Adults & children	427 cases 427 controls	Yes	Overall: Travel outside UK Contact with persons with diarrhoea Touching cattle Toileting contact with a child <5 yrs No. glasses of unboiled water consumed *C. hominis*: Travel abroad, Changing diapers *C. parvum*: Touching farm animals	Overall: Ice cream, raw vegetables and tomatoes *C. hominis*: Sleeping on the ground No. persons 5-15 yrs in household Washing fruit & veg before eating *C. parvum*: Eating raw veg & tomatoes	Drinking water (tap) Swimming pools Age	Hunter *et al.* (2004)

Table A1.1 Contd

Location	Age group	Cases/ Controls	Species data	Risk factors	Protective factors	No Association	Reference
England /Wales	Adults & children	3343 cases 3343 location matched controls	Yes	Overall: Living in area with high *Cryptosporidium* spread on the land Large no. individuals 0-4 yrs living in the area More individuals in high SES bracket *C. parvum*: (As above) *C. hominis*: Living in urban area Area with many in social classes 1-4 Large no. individuals 0-4 yrs living in the area	Overall: Drinking water with superior treatment Drinking water (groundwater) *C. parvum*: Living in urban area Drinking water with superior treatment Drinking water (Groundwater)		Lake *et al.* (2007)
N. Cumbria England	Adults & children	152 cases 466 unmatched controls	Yes (No separate risk factor analysis for species)	Consumption of unboiled tap water in HIV infected patients Visits to farms	Unknown	Pet contact Age Gender Cattle contact	Goh *et al.* (2004)

Table A1.1 Contd.

Location	Age group	Cases/ Controls	Species data	Risk factors	Protective factors	No Association	Reference
United States (7 locations)	Adults & children	282 cases 490 matched controls	No	International travel Cattle contact Contact with persons 2-11 yrs with diarrhoea Freshwater swimming	Eating raw vegetables	Animal exposure Swimming in pools Drinking water sources Gender	Roy *et al.* (2004)

Appendix 2

PCR primers, PCR cycles and RFLP digests used for molecular typing of *Cryptosporidium* oocysts

Table A2.1 Primers for SSU rRNA PCR assay and GP60 subtype analysis

PCR assay		Forward Primer	Forward primer sequence	Reverse primer	Reverse primer sequence	Size PCR product	Reference
DIAG	PCR 1	WR494F	5′ – TGA GTK AAG TAT AAA CCC CTT TAC – 3′	XR1	5′–CCC ATT TCC TTC GAA ACA GGA–3′	~880bp	Ward *et al.* (2002)
	PCR 2	CPB-DIAG.F	5′ – AAG CTC GTA GTT GGA TTT CTG – 3′	CPB-DIAG.R	5′–TAA GGT GCT GAA GGA GTA AGG–3′	~435bp	Johnston *et al.* (1995); Nichols *et al.* (2003)
XIAO	PCR 1	XF1	5′-TTC TAG AGC TAA TAC ATG CG-3′-3′	XR1	5′-CCC ATT TCC TTC GAA ACA GGA-3′	~1,325bp	Xiao *et al.* (1999); Xiao *et al.* (2000); Xiao *et al.* (2001)
	PCR 2	XF2	GGA AGG GTT GTA TTT ATT AGA TAA AG-3′	XR2	5′AAG GAG TAA GGA ACA ACC TCC A-3′	~826bp	
GLAB	PCR 1	AL3531	5′-ATA GTC TCC GCT GTA TTC-3′	AL3535	5′-GGA AGG AAC GAT GTA TCT-3′	~900bp	Glaberman *et al.* (2002)
	PCR 2	AL3532	5′TCC GCT GTA TTC TCA GCC-3′	AL3534	5′-GCA GAG GAA CCA GCA TC-3′	~900bp	
SUL	PCR 1	AL3531	5′-ATA GTC TCC GCT GTA TTC-3′	AL3533	5′-GAG ATA TAT CTT GGT GCG-3′	~900bp	Sulaiman *et al.* (2005)
	PCR 2	AL3532	5′-TCC GCT GTA TTC TCA GCC-3′	LX0029	5′-CGA ACC ACA TTA CAA ATG AAG T-3′	~400bp	

Two-step nested PCR for the *Cryptosporidium* SSU rRNA gene species identification with Diagnostic primers

PCR 1: Reaction volume 50uL

Reagents	**Volume of reagents per tube (µl)**
DNAse free water	15.5
10x PCR buffer IV (ABgene)	5
dNTPs 1/50 (2nM)	5
10x BSA (4mg/ml)	5
Tween 20 (20%)	5
MgCl2 (25nM)	5
PVP 10% pH8	5
Primers (10uM) WR494F	1
XiaoR1	1
Tag polymerase (ABgene)	0.5
Dispense per tube	48
DNA lysate	2

Samples were amplified using thermocycler model 9700 and the following thermocycler program:

1 cycle at 95°C for 5 min
35 cycles of 94°C for 30s
60°C for 30s
72°C for 45s
1 cycle at 72°C for 10 min
Soak at 4°C

PCR 2: Reaction volume 50uL

Reagents	Volume of reagents per tube (µl)
DNAse free water	43.5
10x PCR buffer IV (ABgene)	10
dNTPs 1/50 (2nM)	10
10x BSA (4mg/ml)	10
Tween 20 (20%)	10
MgCl2 (25nM)	10
Primers (10uM) CPB-DIAG.F	2
CPB-DIAG.R	2
Tag polymerase (ABgene)	0.5
Dispense per tube	98
PCR 1	2

Samples were amplified using thermocycler model 9700 and the following thermocycler program:

1 cycle at 94°C for 5 min
35 cycles of 94°C for 30s
60°C for 30s
72°C for 3 min
1 cycle at 72°C for 10 min
Soak at 4°C

10 ul of PCR 2 product was mixed with 4 ul of bromophenol blue (BPB) and 10 ul of each sample were run on 1.4% agarose gel with ethidium bromide.

Two-step nested PCR for the *Cryptosporidium* SSU rRNA gene species identification with Xiao primers

PCR 1: Reaction volume 50uL

Reagents		**Volume of reagents per tube (µl)**
DNAse free water		8.5
10x PCR buffer IV (ABgene)		5
dNTPs 1/50 (2nM)		5
10x BSA (4mg/ml)		5
Tween 20 (20%)		5
MgCl2 (25nM)		12
PVP 10% pH8		5
Primers (10uM)	XF1	1
	XR1	1
Tag polymerase (ABgene)		0.5
Dispense per tube		48
DNA lysate		2

Samples were amplified using thermocycler model 9700 and the following thermocycler program:

1 cycle at 94°C for 3 min
35 cycles of 94°C for 45s
55°C for 45s
72°C for 3 min
1 cycle at 72°C for 7 min
Soak at 4°C

PCR 2: Reaction volume 100uL

Reagents		Volume of reagents per tube (µl)
DNAse free water		41.5
10x PCR buffer IV (ABgene)		10
dNTPs 1/50 (2nM)		10
10x BSA (4mg/ml)		10
Tween 20 (20%)		10
MgCl2 (25nM)		12
Primers (10uM)	XF2	2
	XR12	2
Tag polymerase (ABgene)		0.5
Dispense per tube		98
PCR 1		2

Samples were amplified using thermocycler model 9700 and the following thermocycler program:

1 cycle at 94°C for 3 min

35 cycles of 94°C for 45s
55°C for 45s
72°C for 1 min

1 cycle at 72°C for 7 min

Soak at 4°C

10 ul of PCR 2 product was mixed with 4ul of PBP and 10 ul were run on 1.4% agarose gel with ethidium bromide.

Digestion of Xiao amplicon (~826 bp) PCR 2 product with *Ase*I and separately with *Ssp*I restriction enzyme for *Cryptosporidium* species identification

Digestion mix with *Ase*I restriction enzymes

Reagents	**Volume of reagents per tube (µl)**
DNAse free water	21
10x NPE buffer	5
*Ase*I (20U)	2
PCR 2 product	20

Digestion mix with *Ssp*I restriction enzymes

Reagents	**Volume of reagents per tube (µl)**
DNAse free water	21
React 6 buffer	5
*Ssp*I (20U)	2
PCR 2 product	20

Digestion tubes were incubated at 37°C for 2 hours and run the digest in 2% agarose gel. 50 ul of the digest was mixed with 15ul of BPB and 50 uL were loaded into each well on the gel.

Digestion of Diagnostic amplicon (~435 bp) PCR2 product simultaneously with *Ase*I and *Dra*I restriction enzymes for *Cryptosporidium* species identification

Digestion mix with *Ase*I and DraI restriction enzymes

Reagents	**Volume of reagents per tube (µl)**
DNAse free water	21
10x NPE buffer	5
*Ase*I (20U)	2
*Dra*I (20U)	2
PCR 2 product	20

Digestion tubes were incubated at 37°C for 2 hours and run the digest in 2% agarose gel. 50 ul of the digest was mixed with 15 uL of PBP and 50 ul were loaded into each well on the gel.

Two-step nested PCR for the *Cryptosporidium* GP60 gene subtyping With Glaberman primers

PCR 1: Reaction volume 100 µl

Reagents		**Volume of reagents per tube (µl)**
DNAse free water		31
10x PCR buffer IV (ABgene)		10
dNTPs 1/50 (2nM)		10
10x BSA (4mg/ml)		10
Tween 20 (20%)		10
MgCl2 (25nM)		12
PVP 10% pH8		10
Primers (10uM)	GF1	2
	GR1	2
Tag polymerase (ABgene)		1
Dispense per tube		98
DNA lysate		2

Samples were amplified using thermocycler model 9700 and the following thermocycler program:

1 cycle at 95°C for 3 min
35 cycles of 94°C for 45s
50°C for 45s
72°C for 1 min
1 cycle at 72°C for 10 min
Soak at 4°C

PCR 2: Reaction volume 100 µl

Reagents		**Volume of reagents per tube (µl)**
DNAse free water		41
10x PCR buffer IV (ABgene)		10
dNTPs 1/50 (2nM)		10
10x BSA (4mg/ml)		10
Tween 20 (20%)		10
MgCl2 (25nM)		12
Primers (10uM)	GF2	2
	GR2	2
Tag polymerase (ABgene)		0.5
Dispense per tube		98
PCR 1		2

Samples were amplified using thermocycler model 9700 and the following thermocycler program:

1 cycle at 95°C for 3 min
35 cycles of 94°C for 45s
50°C for 45s
72°C for 1 min
1 cycle at 72°C for 10 min
Soak at 4°C

10 ul of PCR 2 product was mixed with 4ul of PBP and 10ul were run on 1.4% agarose gel with ethidium bromide to test for positives (PCR product ≅ 900 bp) prior to sequencing.

Two-step nested PCR for the *Cryptosporidium* GP60 gene subtyping using Sulaiman primers

PCR 1: Reaction volume 100 µl

Reagents		**Volume of reagents per tube (µl)**
DNAse free water		31
10x PCR buffer IV (ABgene)		10
dNTPs 1/50 (2nM)		10
10x BSA (4mg/ml)		10
Tween 20 (20%)		10
MgCl2 (25nM)		12
PVP 10% pH8		10
Primers (10uM)	GF1	2
	SR1	2
Tag polymerase (ABgene)		1
Dispense per tube		98
DNA lysate		2

Samples were amplified using thermocycler model 9700 and the following thermocycler program:

1 cycle at 95°C for 3 min
35 cycles of 94°C for 45s
50°C for 45s
72°C for 1 min

1 cycle at 72°C for 10 min
Soak at 4°C

PCR 2: Reaction volume 100 µl

Reagents	**Volume of reagents per tube (µl)**
DNAse free water	41
10x PCR buffer IV (ABgene)	10
dNTPs 1/50 (2nM)	10
10x BSA (4mg/ml)	10
Tween 20 (20%)	10
MgCl2 (25nM)	12
Primers (10uM) GF2	2
SR2	2
Tag polymerase (ABgene)	0.5
Dispense per tube	98
PCR 1	2

Samples were amplified using thermocycler model 9700 and the following thermocycler program:

1 cycle at 95°C for 3 min
35 cycles of 94°C for 45s
50°C for 45s
72°C for 1 min

1 cycle at 72°C for 10 min
Soak at 4°C

10 ul of PCR 2 product was mixed with 4ul of PBP and 10ul were run on 1.4% agarose gel with ethidium bromide to test for positives (PCR product ≅ 400 bp) prior to sequencing.

Appendix 3

Risk factors questionnaire

Cryptosporidiosis Investigation Form

Identifying Information

Name: _____

ID Number: _____

Person supplying information (e.g. mother/guardian):

_____

Address: _____

Date of Interview: ___ / ___ / ___

Name of Interviewer: _____

The following questions relate to the child unless otherwise stated

Personal and Household Information

1. Sex: (1) Male / (2) Female

2. Date of Birth: ____ /____ /____

3. Age: _____ months

4. Is the child attending: (1) School ☐

(2) Day Care ☐

(3) Other ☐ Specify _____

5. How many people live in the child's house? Total: _____

(a) No. of Adults (16+ yrs)	(b) No. of Children (5-15 yrs)	(c) No. of Children (<5 yrs)

6. How many families live in the house? _____

7. How many rooms are in the house? _____

Socioeconomic Status

8. Is the house

(1) Owner occupied ☐

(2) Rented ☐

(3) Other ☐ Specify _____

9. Is your house connected to a generator? (1) Yes (2) No

10. Does your house have a working fridge? (1) Yes (2) No

11. Does your house have a television? (1) Yes p No

12. Does your house have a radio? (1) Yes (2) No

13. What type of fuel do you use in your home for cooking most of the time?
Tick one only

(1) Gas ☐

(2) Firewood/coal ☐

(3) Kerosene ☐

(4) Electricity ☐

(5) Other ☐ Specify _____

14. Mobile phone?

(1) Mother ☐

(2) Father ☐

(3) Both ☐

(4) None ☐

15. What is the mother's main occupation?
Tick only one

(1) House/family work *(non-income generating)* ☐

(2) Manual worker ☐

(3) Farmer ☐

(4) Student ☐

(5) Craftsperson (carpenter, Fashion designer) ☐

(6) Business woman ☐

(7) Civil servant ☐

(8) Professional ☐ Specify: _____

(9) Clerk *e.g. secretarial* ☐

(10) Street vendor ☐

(11) Teacher ☐

(12) Other ☐ Specify: _____

16. What is the father's main occupation?
Tick only one

(1) House/family work *(non-income generating)* ☐

(2) Manual worker ☐

(3) Farmer ☐

(4) Student ☐

(5) Craftsperson (carpenter, Fashion designer) ☐

(6) Businessman ☐

(7) Civil servant ☐

(8) Professional ☐ Specify: _____

(9) Clerk *e.g. secretarial* ☐

(10) Street vendor ☐

(11) Teacher ☐

(12) Other ☐ Specify: _____

17. Approximately how much money is available for your household in one month? N_____

18. What is the highest level of education of child's mother?
Tick one only

(1) None ☐

(2) Some primary school (or modern 1) ☐

(3) Completed Primary school ☐

(4) Some 2° school ☐

(5) Some 2° school exit exams ☐

(6) Post – secondary school ☐

19. What is the highest level of education of child's father?
Tick one only

(1) None ☐

(2) Some primary school (or modern 1) ☐

(3) Completed Primary school ☐

(4) Some 2° school ☐

(5) Some 2° school exit exams ☐

(6) Post – secondary school ☐

Medical Information

20. Has the child suffered from any of the following symptoms in the last two weeks?

Symptoms		**Date of Onset**
(a) Diarrhoea *(3 or more loose stools in 24 hours)*	(1)Yes / (2)No	
(b) Vomiting	(1)Yes / (2)No	
(c) Blood in Stools	(1)Yes / 2)No	
(d) Stomach Cramps	(1)Yes / (2)No	
(e) Nausea	(1)Yes / (2)No	
(f) Loss of Appetite	(1)Yes / (2)No	
(g) Fever	(1)Yes / (2)No	
(h) Malaria (As indicated by any Doctor)	(1)Yes / (2)No	

21. (a) Has anyone in your household suffered from similar symptoms in the last two weeks? *(If No, go to Q. 22)*

(1) Yes (2) No

(b) Please give the details below

Number of people ill *(circle)*	**1**	**2**	**3**	**4**	**5**
Age					
Sex					
Relationship with child					
Symptoms					

Water Use

22. (a) Where do you obtain drinking water for your child?
Tick all that apply

(1) Tap inside the house ☐

(2) Tap outside the house (on property) ☐

(3) Shared or community tap
(including fetching water on campus and at water works) ☐

(4) Protected or covered well ☐

(5) Unprotected or uncovered well ☐

(6) Bore hole ☐

(7) River, stream ☐

(8) Other ☐ Specify: _____

23. (a) Do you treat your child's drinking water? (1) Yes (2) No *(If No go to Q. 24)*

(b) If yes, how is the drinking water for your child treated?
Tick all that apply

(a) Boil	(1) Always / (2) Never / (3) Sometimes
(b) Filter	(1) Always / (2) Never / (3) Sometimes
(c) Chlorination	(1) Always / (2) Never / (3) Sometimes
(d) Alum	(1) Always / (2) Never / (3) Sometimes
(e) Allow to settle	(1) Always / (2) Never / (3) Sometimes
(g) Other (Specify)	

(c) Have you treated the water in this way in the last two weeks?

(1) Yes (2) No

24. (a) Where is the child bathed/washed (e.g. stream, public shower etc)? _____

(b) Where do you obtain this bathing water?

(1) Tap inside the house ☐

(2) Tap outside the house (on property) ☐

(3) Shared or community tap
(including fetching water on campus and at water works) ☐

(4) Protected or covered well ☐

(5) Unprotected or uncovered well ☐

(6) Bore hole ☐

(7) River, stream ☐

(8) Other ☐ Specify: _____

Personal Hygiene

25. When does the guardian wash their hands?

Time?	What are they washed with?	Method?	If and how are they dried?
After going to toilet/before eating etc.	Water only, water + soap etc.	Running tap, bowl which others use etc.	Air dried, towel etc.

26. When does the child wash their hands?

Time?	What are they washed with?	Method?	If and how are they dried?
After going to toilet/before eating etc.	Water only, water + soap etc.	Running water, bowl which others use etc.	Air dried, towel etc.

27. (a) What type of toilet do the adults (16+yrs) in the household use most of the time?
Tick only one

(1) Flush toilet ☐

(2) Pit latrine ☐

(3) Ventilated pit latrine ☐

(4) Bush ☐

(5) Potty ☐

(6) Other ☐ Specify: _____

28. (a) What type of toilet does the child use in the household most of the time?
Tick only one

(1) Flush toilet ☐

(2) Pit latrine ☐

(3) Ventilated pit latrine ☐

(4) Bush ☐

(5) Potty ☐

(6) Other ☐ Specify: _____

(b) If a latrine is ever used (by either your child or adults) is this shared with other households?
(1) Yes (2) No *(If No go to Q. 29)*

(c) How many households is the latrine shared with? ☐

29. (a) Does the child wear nappies? (1) Yes (2) No *(If No go to Q. 30)*

(b) How and where are they washed (e.g. basin which is used for other purposes, river etc.)?

_____

(c) Where is the waste from the nappy disposed of?

_____

Food

30. What type of milk does the child drink most of the time?
Tick only one

(a) Soya milk ☐

(b) Powdered milk ☐

(c) Tinned milk ☐

(d) Other ☐ Specify: _____

31. (a) Is water added to this milk? (1) Yes (2) No
(If No go to Q. 32)

(b) Is this water treated before being added to the milk? (1) Yes (2) No
(If No go to Q. 32)

(c) If treated, how is it treated? _____

32. (a) Is the child breastfed at present? (1) Yes (2) No *(If Yes go to Q. 33)*

(b) At what age was your child when you stopped breast feeding? _____(in months)

33. (a) Did you ever breast feed exclusively? (1) Yes (2) No *(If No go to Q. 34)*

(b) For how long?_____(weeks/months)

34. At what age did the child first receive: Water to drink? _____ Months

Solid foods? _____ Months

35. (a) Where do you obtain fruit and vegetables? *(If not grown got to Q. 36)*

(1) Grow your own ☐

(2) Buy at market ☐

(3) Both ☐

(b) If grown, what is used to water and fertilise the plants?
Tick all that apply

(1) River water ☐

(2) Borehole/Well ☐

(3) Animal manure ☐

(4) Human waste ☐

(5) Chemicals ☐

(6) Other ☐ Specify: _____

36. (a) Are fruit and vegetables washed before eaten? (1) Yes (2) No

(If No go to Q. 37)

(b) What are they washed with?

(1) Treated water ☐ Specify treatment: _____

(2) Untreated water ☐ Source: _____

Animal Contacts

37. (a) Do you keep domesticated animals? (1) Yes (2) No

(If No go to Q. 38)

(b) If yes, what animals do you keep?
Tick all that apply

(1) Dog ☐
(2) Goat ☐
(3) Cow ☐
(4) Cat ☐
(5) Chicken ☐
(6) Others ☐ Specify: _____

38. Which of the following animals is the child in close contact with regularly?
Tick all that apply

(1) Dog ☐
(2) Goat ☐
(3) Cow ☐
(4) Cat ☐
(5) Chicken ☐
(6) Others ☐ Specify: _____
(7) None ☐

Malaria

49. Use of Mosquito Nets?

	(1)Yes	(2)No		
(a) Window net				
(b) Door Net			Ordinary	Insecticide treated nets
(c) Bed Net				

THANK YOU FOR YOUR TIME AND FOR TAKING PART IN THIS QUESTIONNAIRE

Appendix 4

Interviewer's training document

Interviewer Training

Date: Wednesday 8th August 2007

Proposed Interviewers: Miss Olademiji Obasa, Miss Olaremi Olafiaji, Mr. Mayowa Adesina, Miss Damilola Omigade, Mr. Seye Jegede

The Interviewer's responsibilities

The interviewer's job is to see that the interview is completed honestly and accurately according to the instructions specified during training. Specifically this means:

- Clearly understanding the nature and content of the questions before starting the interview.
- Being familiar with all instructions.
- Making sure the interview is conducted with the correct respondent.
- Recording exact details. This means neither adding nor deleting any information and executing the work clearly and accurately.
- Striving for maximum efficiency without sacrificing quality.
- Being courteous and friendly.

Basic Interviewing Rules

- Read all the questions word for word.
- Ensure that ALL questions are answered. Answers which remain blank will seriously compromise the study and analysis of information will be unable to be performed.
- Read all questions in the exact order in which they appear.
- Do not skip appropriate questions even if you feel you know the answer.
- Never hurry the interview.
- Remain objective. Do not indicate surprise, pleasure or disapproval at any respondent's answers.
- Be prepared to probe on some answers.