SIGNIFICANCE OF INTESTINAL PROTOZOAN PARASITES AS DIARRHOEA-CAUSING INFECTIOUS AGENTS IN CHILDREN PRESENTING TO THE AGOGO PRESBYTERIAN HOSPITAL

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DECLARATION

I hereby declare that this submission is my own work towards the MPhil and that, to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any other degree of the University, except where due acknowledgement has been made in the text.

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ABSTRACT

Diarrhoeal disease is one of the leading causes of morbidity and mortality in young children in developing countries. This study assessed the use of multiplex real-time PCR assay and microscopy to determine the prevalence and significance of intestinal protozoan parasites as diarrhoeal-causing infectious agents in children in the Ashanti Akyem North District of the Ashanti Region, Ghana. Between June 2007 and May 2008, fresh stool samples were obtained from 1373 children up to 13 years of age attending the child welfare clinic of the Agogo Presbyterian Hospital. 0.20g of each stool samples collected was preserved at -20^{0}C for DNA extraction whilst the remaining was preserved in sodium acetate-acetic acid formalin and concentrated using the formol-ether technique for microscopic examination. DNA extracts were analyzed with the multiplex real time Polymerase Chain Reaction (RT-PCR) for pathogenic protozoan parasites. Of the 1373 children examined, 60.5% were clinically declared symptomatic whilst 39.5% were asymptomatic. The prevalence rates of the enteric protozoa detected by microscopy from the symptomatic and asymptomatic children were *Giardia lamblia*, 13.1% and 20.5%, *Cryptosporidium* species, 4.9% and 4.9%, *Entamoeba histolytica/dispar*, 0.4% and 1.2%, *Blastocystis hominis*, 10.1% and 20.3% *Entamoeba coli,* 4.0% and 12.7%, *Chilomastix mesnili*, 2.6% and 6.2%, *Endolimax nana* 1.8% and 8.7%, *Entamoeba hartmani* 0.68% and 3.32% respectively. The prevalence rates of helminths detected in both symptomatic and asymptomatic children were *Ascaris lumbricoides* 0.16%, Hookworm 0.6%, *Strongyloides stercolaris* 1.0%, *Hymenolepis nana* 0.4% and *Metagonimus* species 0.5%. Multiplex real time PCR detected 31.2% *Giardia lamblia* and 5.7% *Cryptosporidium parvum* in the symptomatic whilst 40.3% *Giardia lamblia*, 2.4% *Cryptosporidium parvum* and only one case of *Entamoeba histolytica* were detected in the asymptomatic children. By using an expanded gold standard the sensitivity and specificity of PCR for *Giardia lamblia* detection was 96.9% and 81.6% respectively, whilst 65.2% sensitivity and 98.7% specificity was observed with microscopy. Sensitivities of 76.2% and 80% and specificities of 98.2% and 97.2% for microscopy and PCR respectively for the detection of *Cryptosporidium parvum* were also observed. This present study showed low rates of helminths and relatively high rates of protozoa infections in the study children. However, protozoa pathogens detected amongst the symptomatic and asymptomatic children were found to be similar; thus the significance of these pathogens as diarrhoeal causing agents in the district is therefore unclear. This present study has also demonstrated that the multiplex real time PCR assay was more sensitive compared to microscopy in the diagnosis of the intestinal protozoa parasites.

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INTRODUCTION

1.1 General introduction

In spite of the progress made in medical research that has produced drugs against infectious agents-bacteria, viruses, fungi and parasites – infectious diseases caused by these agents are still a major cause of socio-economic disturbances, disability and death for millions around the world (Guerrant *et al.*, 1990). These diseases – lower respiratory infections, HIV/AIDS, diarrhoeal diseases, tuberculosis, malaria and measles - are also the leading causes of death in sub-Saharan Africa (WHO, 2000).

Diarrhoea diseases leading to childhood malnutrition, morbidity and mortality (Guerrant *et al.*, 1990) cause 1.8 million deaths annually worldwide among children under five years (WHO, 2006), 80% occurring in the first two years of life (WHO, 1998). Diarrhoeal diseases rank third to lower respiratory infections and HIV/AIDS as leading cause of death due to infectious diseases (WHO, 2004). It is estimated that 1.5 billion episodes of diarrhoea occur annually worldwide among children under five (WHO, 2003). On average, children below three years of age in developing countries experience three episodes of diarrhoea each year. These episodes of diarrhoea have been found to have adverse effect on growth (Bhutta and Hendricks, 1996). In Ghana, morbidity due to diarrhoea cases reported over the past five years ranged from 3.4% to 4.3% (MoH, 2006). The 2006 and 2007 morbidity due to diarrhoea in the Ashanti region as reported by the regional health directorate was 4.9% and 3.6% respectively. The Ashanti Akyem North District (AAND) in the Ashanti Region, reported morbidity ranged from 3.40% to 4.0%. The Agogo Presbyterian Hospital recorded rates of 3.2% (2006) and 4.2% (2007).

Diarrhoea is defined as the passage of loose, watery stools occurring more than three times in one day (WHO, 1997b). There are four clinical types of diarrhoeal

diseases: acute watery diarrhoea, acute bloody diarrhoea (dysentery), persistent diarrhoea and diarrhoea with severe malnutrition - marasmus or kwashiorkor (Lima and Guerrant, 1992). Although the etiologic and pathophysiologic factors of diarrhoea may be caused by nutritional deficiencies (Lima and Guerrant, 1992) and food allergy (Walker-Smith, 1984), the ultra structural derangements of the small intestinal mucosal surfaces observed by the scanning electron microscope has shown an association of enteropathogens acting directly on the enterocytes (Fagundes-Neto *et al.*, 1997) that elicit the diarrhoea process (Fagundes-Neto *et al.*, 2000).

Entamoeba histolytica, Giardia lamblia and *Cryptosporidium parvum* are three of the most common intestinal protozoan parasites infecting humans worldwide. They are known to be the most important diarrhoea-causing protozoa (Marshall *et al.*, 1997). It is estimated that 10% of the world's population is infected with E. histolytica with the highest prevalence in developing countries (Vandenberg *et al.*, 2006). Global statistics on the prevalence of *E. histolytica* infection indicates that 90% of infected individuals remain asymptomatic carriers while the other 10% develop clinically apparent disease. This results in 50-100 million cases of colitis or liver abscesses per year and up to 100,000 deaths annually (Ayeh-Kumi *et al.*, 2001). Rates of 20-40% of the global burden *of G. lamblia* are reported in developing countries, especially in children. Prevalence of cryptosporidiosis in Asia and Africa ranges from 5-10% (Vandenberg *et al.*, 2006). The intestinal protozoa organisms may be common worldwide, however, the prevalence rate is higher in developing countries. Their frequency may be related to the inadequacy of sanitation, water supply, healthcare, education and poverty.

Laboratory diagnosis of these protozoan parasites for many years has relied on the traditional microscopic examination of stool samples. This is regarded as the gold standard when performed by an experienced and a highly skilled microscopist (Weiss, 1995). However, the sensitivity and specificity of the microscopic

technique has been found to be rather low (Haque *et al.*, 1998; Petri *et al.*, 2000) . It is laborious and requires long professional training and may present false positive and negative results. The principal limitation of this method is its inability to differentiate closely related species and heterogeneity within species, as it is often difficult to differentiate cysts of the pathogenic from the non-pathogenic intestinal protozoa (Petri *et al.*, 2000).

To optimize parasite detection and identification, other diagnostic methods have been developed such as the Immunofluorescence (IF), Enzyme-linked immunosorbent assay (ELISA), culture and subsequent differentiation by isoenzyme analysis and the Polymerase Chain Reaction (PCR) (Garcia and Shimizu, 1997; Nunez *et al.*, 2001). These have been introduced as alternative methods that are more sensitive and specific (Garcia and Shimizu, 1997). These applications however, also have limitations. Their sensitivity and specificity ranges from 66.3% to 100% and 92.6% to 100% respectively (Garcia *et al.*, 2000). The traditional PCR protocols require further processing of the amplicon, which is time-consuming and prone to false-positive results due to possible cross-contamination (Wittwer *et al.*, 1997). In an effort to improve on the PCR protocol, the multiplex real-time PCR has been developed which is able to circumvent the problems associated with the traditional PCR and the other detection methods. This method allows specific detection of the amplicon, discriminating between *E. histolytica, E. dispar, G. lamblia*, and *C. parvum* in a single assay by binding to one or two fluorescence-labeled probes during PCR. Thus, additional analysis of the amplicon is not required. This reduces the time needed to obtain results. It is capable of detecting the minimum amounts of organisms required to cause disease and the presence of multiple protozoan species in a single clinical sample. In addition, the closed reaction tube minimizes the potential for cross-contamination, and the assay output is quantitative (Blessmann *et al.*, 2002; Qvarnstrom *et al.*, 2005). This improves the diagnosis of parasitic diarrhoeal infection, hence patient management.

1.2 Statement of problem

Despite the implementation of preventive measures and technological advances in many parts of the world to minimize the prevalence of parasitic diseases, intestinal protozoa and helminths still account for a rise in child growth retardation, morbidity and mortality in developing countries (WHO, 1986). Poor performance characteristics of traditional diagnostic procedure in many laboratories have resulted in the misdiagnosis of the intestinal protozoa. The consequence is that there is mistreatment and the promotion of drug resistance. Furthermore, the incidence and the prevalence as well as the exact morbidity and mortality rates caused by these pathogens are not appropriately ascertained. The need for new methods that will circumvent the problems associated with the traditional methods is therefore apparent.

1.3 Justification

The morbidity and mortality associated with intestinal protozoa infections coupled with the recent identification of *Entamoeba dispar* as a separate non-pathogenic species which is morphologically indistinguishable from *E. histolytica* and does not require treatment has suggested the need of alternative detection methods (Diamond and Clark, 1993). Molecular methods such as the traditional PCR have been proven to be considerably more sensitive than the microscopy methods and have helped in circumventing the limitations associated with microscopy. Moreover the multiplex closed-tube Real-time PCR which is an improvement of the traditional PCR allows the simultaneous detection of the amplicon of *E. histolytica*, *E. dispar*, *G. lamblia* and *C. parvum* in a single assay by binding to one or two fluorescence-labeled probes during PCR. This reduces the time needed to obtain results. It is capable of detecting the minimum numbers of organisms required to cause disease, and the presence of multiple protozoan species in a single clinical sample (Verweij *et al.*, 2004). In addition, the closed reaction tube

minimizes the potential for cross-contamination, and the assay output is quantitative rather than qualitative (Blessmann *et al.*, 2002).

This assay provides rapid and accurate results and has the capacity for pathogen detection and genotyping. It differentiates between the main species of organisms thereby allowing appropriate epidemiological studies to be conducted and to assist in the assessment of accurate and appropriate interventions, prevention and control. This will also provide clear information on the changing distribution of the main species within the human population and the environment. Since the epidemiology of the main species of the intestinal protozoa may be different both in reservoirs, transmission, age distribution and seasonality, it would therefore be justifiable to apply this technique.

1.4 General objective

The objective of this work is to determine the prevalence and the significance of intestinal protozoa as causative agents of diarrheal diseases among children in the Ashanti Akyem North District of the Ashanti Region.

1.5 Specific objectives

1. To study the prevalence of *Entamoeba histolytica, Giardia lamblia* and *Cryptosporidium parvum* in faecal samples of study children in the district.

2. To determine the distribution of the protozoa parasites in the study children from the sub districts.

3. To compare the prevalence of *E. histolytica, G. lamblia* and *C. parvum* in faecal samples in children using microscopy and Real time PCR diagnostic methods.

4. To compare the test performance characteristics of microscopy and real time PCR to an expanded gold standard in the diagnosis of protozoa parasites in faecal samples of children.

5. To assess co-morbidity of infections of the protozoan parasites in the study children.

Chapter 2

LITERATURE REVIEW

2.1 Diarrhoeal diseases

Diarrhoeal diseases are important causes of childhood morbidity and mortality in developing countries and a principal cause of malnutrition (Guerrant *et al.*, 1990). Diarrhoeal diseases are usually associated with symptoms such as abdominal cramps, nausea, vomiting, fever, malaise and dehydration. Stools of patients with diarrhoea may contain blood or mucus. However, it is the consistency of the stools rather than the frequency that is most important (WHO, 1997b).

2.2 Clinical types of diarrhoeal diseases

There are four clinical types of diarrhoea recognized by the world health organization, each reflecting the basic underlying pathology and altered physiology (WHO, 1997b). (i) Acute watery diarrhoea (including cholera), which lasts several hours or days: the main danger is dehydration; weight loss which occurs if supportive treatment is not given. (ii) Acute bloody diarrhoea, which is also called dysentery; the main dangers are intestinal damage, sepsis and malnutrition and other complications, including dehydration. (iii) Persistent diarrhoea, which lasts 14 days or longer; the main danger is malnutrition and serious non-intestinal infection. (iv) Diarrhoea with severe malnutrition (marasmus or kwashiorkor): the main dangers are severe systemic infection, dehydration, heart failure and vitamin and mineral deficiency (WHO, 1997b).

2.3 Diarrhoea causing pathogens

The important enteropathogens causing diarrhoea are Bacteria (e.g. *Enterotoxigenic Escherichia coli* and other obligate pathogenic *E.coli, Campylobacter jejuni, Vibrio cholera, Yersinia species, Shigella species, Salmonellae, cytotoxigenic Clostridium difficile),* Viruses (e.g. *Rotavirus, Noro viruses and Enteric adenoviruses*) and Parasites such as the Helminths (e.g. *Strongyloides lumbricoides*) and Protozoa (e.g. *Entamoeba*

histolytica, Giardia lamblia and *Cryptosporidium parvum, Blastocystis hominis, Isospora respectively*) (Guerrant *et al.*, 1990).

Parasitic infections are common generally worldwide, but the single-celled protozoa parasites are more prevalent in developing countries. There are four (4) broad groups of intestinal protozoa; the Amoebae, Flagellates, Ciliates and the Coccidia (Levine *et al.*, 1980).

2.4 The intestinal flagellates

There are several intestinal flagellates; *Chilomastix mesnili, Retortamonas intestinalis, Enteromonas hominis* and *Trichomonas hominis* that infect man. However *Giardia lamblia* is the only intestinal flagellate that is considered to be pathogenic(Washington Winn *et al.*, 2006)

2.5 *Giardia lamblia*

G lamblia is the most commonly isolated intestinal parasite throughout the world. Prevalence rates of 20-40% are reported in developing countries, especially in children (Fraser, 1994). Analysis of 722 faecal DNA samples revealed that a prevalence rate of 9.3% of *G. lamblia* by PCR, as compared to 5.7% by microscopy in the Netherland (ten Hove *et al.*, 2007). From a total of 480 patients and apparently healthy Egyptian children selected, the prevalence rate of *G. lamblia* infection detected by concentration-sedimentation method was 11.0% (El-Naggar *et al.*, 2006). Giardiasis (21.1%) was the commonest infections observed when 298 stools specimens were examined in Khartoum, Sudan in a community based prospective study conducted among randomly selected children aged less than five years (Karrar and Rahim, 1995). In a study conducted in northern Ghana by Klaus *et al.*,(2007), *Giardia lamblia* were observed more than twice as frequently in asymptomatic individuals 12(9.7%) than in symptomatic individuals 9(3.7%). The diplomad *Giardia* exist in two morphological forms, the trophozoite and the cyst. The trophozoite is easily recognized under a microscope. It is about 12 to 15 µm long, shaped like a pear cut in half lengthwise, and has two nuclei, structures

called median bodies, and four pairs of flagellae. The flagellae help these organisms to migrate to a given area of the small intestine, where they attach by means of an adhesive disk to epithelial cells and thus maintain their position despite peristalsis. *Giardia* cyst - the form usually seen in the faeces, is ovoid, 6 to 12 µm long, and can often be seen to contain two to four nuclei at one end and prominent diagonal fibrils (Meyer, 1990).

2.5.1 Multiplication and life cycle of *Giardia*

The trophozoite, the actively metabolizing, motile form, lives in the duodenum and jejunum and multiplies by binary fission. Trophozoites that are swept into the faecal stream lose their motility, round up, and are excreted as dormant, resistant cysts. Excreted trophozoites disintegrate. The cyst is sufficiently hardy to survive host-to-host transfer. *Giardia* infection is acquired by ingestion of mature cysts (infective dose varies from 10-100 cysts) via contaminated water or food. The exposure of cysts to host stomach acidity and body temperature triggers excystation, with the emergence of trophozoites which rapidly multiply and attach to the host small intestinal villi by means of a disk on their posterior or ventral surface (Meyer, 1990).

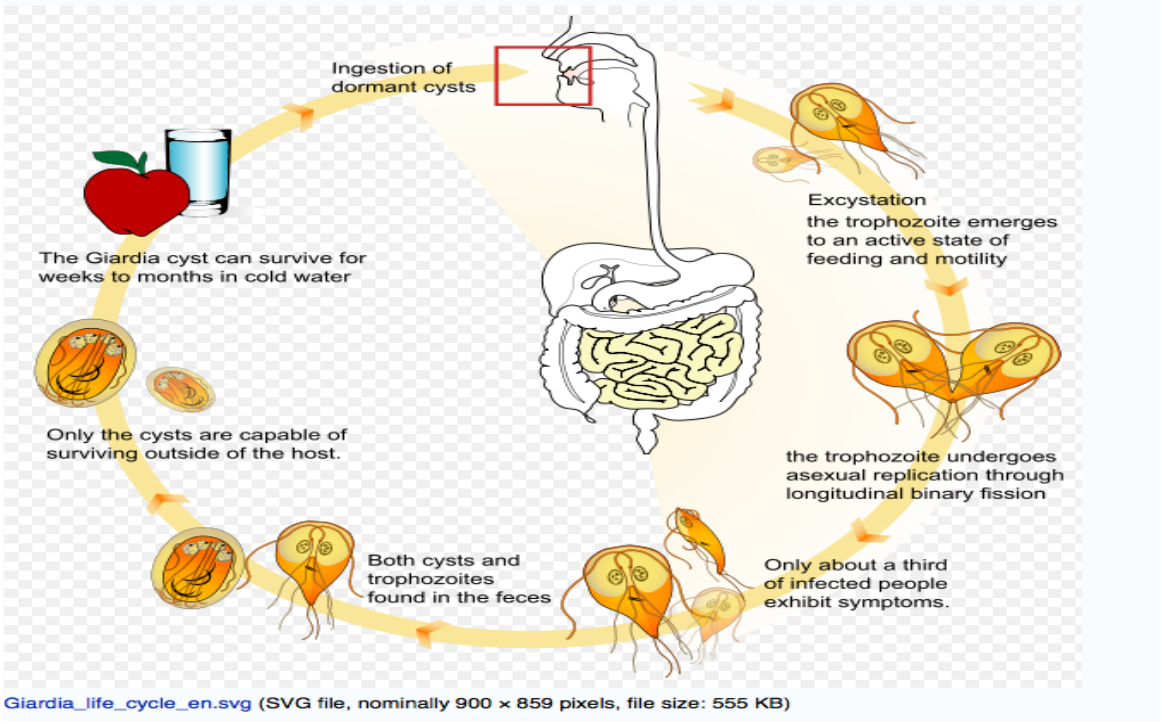


Figure 2.1: Life cycle of *Giardia lamblia*

Source: http://en.wikipedia.org/wiki/File:*Giardia*_life_cycle_en.svg

2.5.2 Pathogenesis of *Giardia*

The pathogenesis of diarrhoea in giardiasis is thought to be related to the following factors: (1) the number of organisms ingested, (2) the specific strain ingested, (3) non antibody protective factors in the gastrointestinal tract, and (4) the immune response of the host (Meyer, 1990). Lectin, a protein on the trophozoite lining, recognizes specific receptors on the intestinal cell and may be partly responsible for the tight attachment between the parasite and the villi, which is followed by mucosal damage, mechanical obstruction (only caused in the presence of numerous organisms), and deconjugation of bile salts (Washington Winn *et al.*, 2006). Studies have indicated that inflammatory mast cells may interfere with duodenal growth of *G. lamblia* trophozoites. Other inflammatory cells, as well as CD8+ T cells, contribute to villus-shortening and crypt hyperplasia. Inflammation results in an increased turnover rate of intestinal mucosal epithelium. The

immature replacement cells have less functional surface area and less digestive and absorptive ability (Meyer, 1990).

2.5.3 Clinical manifestations of *Giardia lamblia* infection

Giardia infection may be asymptomatic or it may cause disease ranging from self-limiting diarrhoea to a severe chronic syndrome (Meyer, 1990). The length of the incubation period, usually 1 to 3 weeks, depends at least partly on the number of cysts ingested. Normal human hosts with giardiasis may have any or all of the following signs and symptoms: loose, foul-smelling stools, steatorrhea (fatty diarrhea), malaise, abdominal cramps, excessive flatulence, fatigue and weight loss or a coeliac-disease-like syndrome (Washington Winn *et al*., 2006). Although most cases are seen in hosts with some concurrent condition, such as an immune deficiency, protein-calorie malnutrition, or bacterial overgrowth of the small intestine, some cases of severe giardiasis occur in apparently normal hosts. Different strains of *G lamblia* possibly vary in virulence (Smith and Wolfe, 1980).

2.5.4 Host defense to *Giardia lamblia* infection

The fact that many *Giardia* infections in humans and experimental animals resolve spontaneously implies that an effective host immune response develops (Heresi and Cleary, 1997). There is evidence that both humoral and cellular immune mechanisms are involved. Hosts with gamma globulin deficiencies are prone to severe disease, suggesting that humoral immunity plays a role. *In vitro* studies have found that normal human milk (but not cow milk or goat milk) kills trophozoites of both *G. lamblia* and *Entamoeba histolytica,* and that this killing does not depend on secreted IgA. This finding raises the possibility that even mother's milk that does not have antiprotozoal antibody may protect infants exposed to these parasites (Meyer, 1990).

2.6 The *Coccidia*

The Coccidia are small protozoa within the subphylum sporozoa. The important intestinal coccidia include *Cryptosporidium parvum* and *Isospora belli*. They are obligate tissue parasites with sexual and asexual stages in their life cycle (Washington Winn *et al*., 2006).

2.7 *Cryptosporidium parvum*

Cryptosporidia are intracellular parasites, occurring in large numbers in small intestinal mucosal epithelial cells. The oval oocyst (the form passed in the feces) is 4µm to 5µm long and may be seen to contain four sporozoites (Navin and Juranek, 1984; Current and Owens, 1989). *Cryptosporidium* infection is common worldwide, and its frequency may be related to the inadequacy of sanitation. In general, the infections are more common in developing countries (Meyer, 1990). Prevalence of *Cryptosporidium* in Asia and Africa ranges from 5-10% (Current and Garcia, 1991). However higher rates of 27.8% and 15.6% from 227 children with diarrhoea and 77 children without diarrhoea respectively, aged less than 5 years have been reported in Ghana (Adjei *et al*., 2004). In another study in Kumasi Ghana, analysis of 474 acute phase stool samples collected from children 2 months - 5 years of age found 61 (12.9%) rate of infection (Addy and Aikins-Bekoe, 1986). Meanwhile analysis of 4,899 samples in Kenya showed an overall prevalence of 4% (Gatei *et al*., 2006). In the industrialized countries it is estimated that at least 325 water-associated outbreaks of this parasitic protozoan disease have been reported. North America and Europe accounted for 93% of all reports, and nearly two thirds occurred in the United States (Karanis *et al*., 2007). ten Hove *et al*., (2007) observed a strong association of Cryptosporidiosis with age when they detected by PCR that 21.8% of 110 children aged <5 years were infected in the Netherlands.

2.7.1 Multiplication and life cycle of *Cryptosporidium*

Cryptosporidium species live inside the epithelial cells of within the intestinal tract of a variety of vertebrates, but some species can infect the respiratory tract (Meyer, 1990). Infection starts with ingestion of an oocyst containing four sporozoites. The

excystation procedure usually requires reducing conditions. Digestive enzymes such as pancreatic enzymes and bile salts trigger the release of sporozoites from the oocyst; however, it is possible for *Cryptosporidium* oocysts to exist in warm aqueous solutions without any special stimuli (Sterling and Arrowood, 1986). Sporozoites enter epithelial cells, taking up a position that is inside the cell (intracellular) but outside the cytoplasm (extra-cytoplasmic). They develop as trophozoites and undergo an asexual multiple budding process (schizogony) to produce a schizont, which releases merozoites.

Merozoites invade other epithelial cells, develop into trophozoites and also undergo schizogony to release more merozoites. Merozoites of this second cycle of asexual reproduction infect further epithelial cells but mature into either 'male' (microgamonts) or 'female' (macrogamonts) gametes to begin the sexual part of the life cycle. Microgamonts produces microgametes which, on release, fertilize the macrogamont to produce a zygote. The zygote may follow two different oocysts generating developmental routes. It can either transform into an oocyst by secretion of a thick wall and development of sporozoites, or into a thin walled oocyst containing sporozoites. Thin walled oocysts are able to liberate sporozoites, thus allowing further rounds of asexual reproduction within the host (Mahon and Manuselis, 2000). Approximately 20% of the zygotes develop into thin-walled oocysts, which represent auto infective life cycle forms that can maintain the parasite in the host. *Cryptosporidium* is an obligate parasite and cannot grow outside the body except in tissue culture (Current and Garcia, 1991; Slifko *et al.*, 2002).

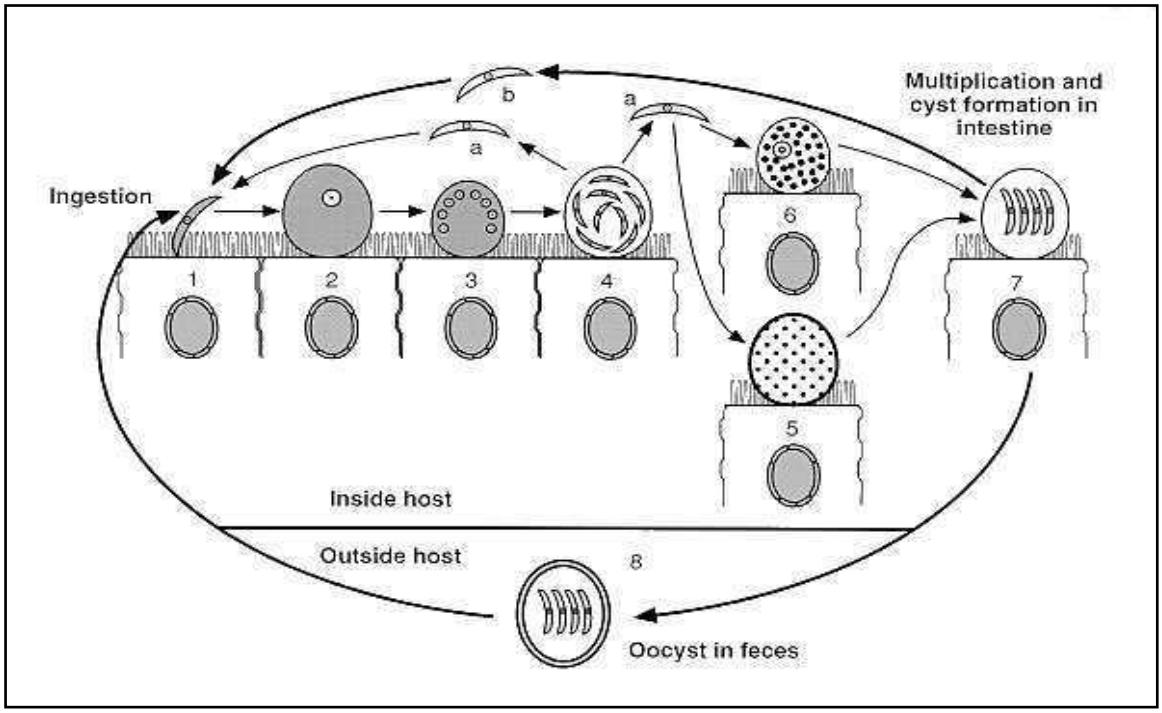


Figure 2.2: Life Cycle of *Cryptosporidium parvum*
Source: Microbiol Rev 47:84, (1983).

(1-4) Asexual cycle of the endogenous stage: (1) sporozoite or merozoite invading a microvillus of a small intestinal epithelial cell; (2) a fully grown trophozoite; (3) a developing schizont with eight nuclei; (4) a mature schizont with eight merozoites. (5, 6) Sexual cycle; (5) microgametocyte with many nuclei; (6) macrogametocyte. (7) A mature oocyst containing four sporozoites without sporocyst. (8) Oocyst discharged in the feces. (a) Merozoite released from mature schizont; (b) sporozites released from mature oocyst. (Reproduce from Tzipori S: Cryptosporidiosis in animals and humans.

2.7.2 Pathogenesis of *Cryptosporidia*

After invasion of the enterocytes, the epithelial cells release cytokines. These cytokines activate phagocytes and recruit new leukocytes, which, in turn, release soluble factors (resulting in intestinal secretion of chloride and water) and inhibit absorption (Wichro *et al.*, 2005). Enterocyte damage may be a direct consequence of parasite invasion, multiplication, and extrusion. Regardless of the specific mechanism, marked distortion of the villus architecture is accompanied by nutrient malabsorption and osmotic diarrhoea (Mahon and Manuselis, 2000; Hunter *et al.*, 2004). Examination of biopsy material from symptomatic patients has revealed a variety of changes in intestinal mucosa, including partial villous

atrophy, crypt lengthening, low cuboidal surface epithelium, cellular infiltration of the jejunal and ileal lamina propria, and inflammation (Washington Winn *et al.*, 2006). Although host cells are damaged in cryptosporidiosis, the means by which the organism causes damage is not known (Blanshard *et al.*, 1992). Mechanical destruction and the effects of toxins, enzymes, or immune-mediated mechanisms, working alone or together may be instrumental (Osewe *et al.*, 1996). More severe cases of cryptosporidiosis are observed in patients with AIDS who have very low CD4 counts <50-100/μL (Mahon and Manuselis, 2000).

2.7.3 Host defenses to *Cryptosporidia*

The fact that cryptosporidiosis is self-limiting in normal persons but usually severe and long-lasting in lower animals and immunocompromised humans indicates that host immune mechanisms are probably involved in eliminating the parasite (Washington Winn *et al.*, 2006). Cell-mediated immunity appears to be the primary defense mechanism. Serum antibodies to *Cryptosporidium* have been shown to develop during recovery from infection (Meyer, 1990). The importance of cryptosporidiosis in immunocompromised patients generally, and in AIDS patients in particular, should not be underestimated. In this group, cryptosporidiosis can cause diarrhoea that is severe, prolonged, and life-threatening. In cryptosporidiosis patients who are immunosuppressed because of drug treatment, the disease can be reversed by withdrawing the drug. Unfortunately, this cure cannot be resorted to with AIDS patients (Navin and Juranek, 1984; Washington Winn *et al.*, 2006).

2.8 The intestinal Amoebae

Three genera of amoeba may inhabit the intestinal tract of humans: *Entamoeba, Iodamoeba and Endolimax.* Members of these genera considered non-pathogenic include *Entamoeba hartmani, Entamoeba gingivalis, Entamoeba coli, Endolimax nana*, and *Iodamoeba butschlii* (Mahon and Manuselis 2000; (Washington Winn *et al.*, 2006).

2.8.1 Classification of the Amoebae

Morphologically identical amoebae may be identified as pathogenic or non-pathogenic on the basis of size, cultural characteristics, virulence in a rat model or in tissue culture, selective agglutination by lectins (Haque *et al.*, 2000), reaction with monoclonal antibodies, or isoenzyme patterns (Jackson and Suparsad, 1997). A pathogen-specific galactose adhesion epitope is described (Haque *et al.*, 2000). Ribosomal RNA sequence analysis and restriction fragment length polymorphism analysis also can separate pathogenic from non-pathogenic strains (Zaki and Clark, 2001). Two classic tests to identify pathogenic strains are the ability to cause cecal ulceration in weanling rats and agglutination by the lectin concanavalin A. These tests of virulence have been supplanted by isoenzyme analysis and the use of monoclonal antibodies to identify pathogenic strains of *E histolytica* (Gonin and Trudel, 2003). Isoenzyme patterns are known for four amebic enzymes: glucose phosphate isomerase (GPI), hexokinase (HK), malate: NADP+ oxidoreductase (ME), and phosphoglucomutase (PGM). The isoenzyme patterns of three of these, GPI, HK, and PGM, can be used to define 20 zymodemes of *E. histolytica* (Bracha *et al.*, 1990). Zymodemes II, VI, VII, XI, XII, XIII, XIV, XIX and XX are pathogenic. Zymodemes II and XI are responsible for liver abscesses (Sargeaunt *et al.*, 1980; Sargeaunt *et al.*, 1984; Mirelman *et al.*, 1986). However, critical reanalysis of *E. histolytica* strains previously typed by Sargeaunt and colleagues demonstrated the existence of only two zymodemes of *E. histolytica,* with many of the original zymodeme patterns being due to contaminating bacterial isoenzymes (Ayeh-Kumi *et al.*, 2001).

2.8.2 *Entamoeba histolytica*

This parasite was named for its remarkable ability to lyse human tissues. A requirement to amoebic invasion is the parasite's ability to colonize and penetrate colonic mucins overlying the intestinal epithelium (Sodeman and William, 1990). *Entamoeba histolytica* infection is one of the most common parasitic infections worldwide, infecting about 50 million people, often in developing countries,

resulting in 40,000 to 100,000 deaths per annum It has long been known that although about 500 million people each year have amoebiasis, only about 10% experience symptomatic disease (WHO, 1997a; Ayeh-Kumi *et al*., 2001). It is probable that 90% of the infections previously ascribed to *E. histolytica* are actually *E. dispar*, while only the remaining 10% are *E. histolytica* infections (Zaki and Clark, 2001). Mora *et al.,* (2008) found that the *E. histolytica/E. dispar* prevalence rates according to the direct, Ritchie and trichromic staining methods were 20.09%, 13.79% and 12.15%, respectively; while prevalence rates according to PCR for *E. histolytica* and *E. dispar* were 6.31% and 4.44%, respectively, in a study they conducted in Venezuela. Direct stool examination from 134 individuals with diarrhoea detected *E. histolytica/E. dispar* in eight (6%) whiles analysis by PCR showed *E. dispar* in ten (7.5%) and *E. histolytica* in two cases (1.5%) in a related study conducted in Nicaragua (Leiva *et al*., 2006). In northern Ghana, Verweij *et al*., (2003) showed a high prevalence (39.8%) of *E. histolytica/dispar* complex by microscopy but 82.8% of *Entamoeba dispar* and only one case of *Entamoeba histolytica* by PCR.

2.8.2.1 Structure of the *Entamoeba histolytica*

E. histolytica has a relatively simple life cycle that alternates between trophozoite and cyst stages The trophozoite is the actively metabolizing, mobile stage and the cyst is dormant and environmentally resistant (Washington Winn *et al*., 2006). Diagnosis of the infection is focused on both stages. Trophozoites vary remarkably in size-from 10 to 50 μm in diameter and when they are alive they may be actively motile (Mahon and Manuselis, 2000). The amoebae are anaerobic organisms which do not have mitochondria. The finely granular endoplasm contains the nucleus and food vacuoles, which in turn may contain bacteria or red blood cells. The parasite is sheathed by a clear outer ectoplasm. The nucleus has a distinctive central karyosome and a rim of finely beaded chromatin lining the nuclear membrane (Sodeman and William, 1990). The cyst is a spherical structure, 10-20 μm in diameter, with a thin transparent wall (Sodeman 1990). Fully mature cysts

contain four nuclei with the characteristic amoebic morphology. Rod-like structures (chromatoidal bars) are present variably, but are more common in immature cysts. Inclusions in the form of glycogen masses also may be present (Beaver *et al.*, 1984).

2.8.2.2 Multiplication and life cycle of *Entamoeba histolytica*

The fecal-oral transmission of amoeba usually involves contaminated food or water. The parasite can also be transmitted directly by ano-genital or oro-anal sexual contact (Washington Winn *et al.*, 2006). Latent infections can become invasive in a setting of impaired host immunity (Meyer, 1990). The Amoebae multiply in the host by simple binary fission. Most multiplication occurs in the host, and survival outside the host depends on the desiccation-resistant cyst form. Encystment occurs apparently in response to desiccation as the amoeba is carried through the colon. After encystment, the nucleus divides twice to produce a quadrinucleate mature cyst. Mature cysts are ingested via contaminated water or food. Excystment occurs after ingestion and is followed by rapid cell division to produce four amoebae which undergo a second division in the small intestine. Trophozoites inhabit the large intestine and can either invade the tissue (pathogenic amoeba) or are eliminated in the stools. Trophozoites do not survive outside the body (Enrique Chacon-cruz and Douglas Mitchell, 2007). Each cyst yields eight tiny amoebae (Mahon and Manuselis, 2000; Washington Winn *et al.*, 2006).

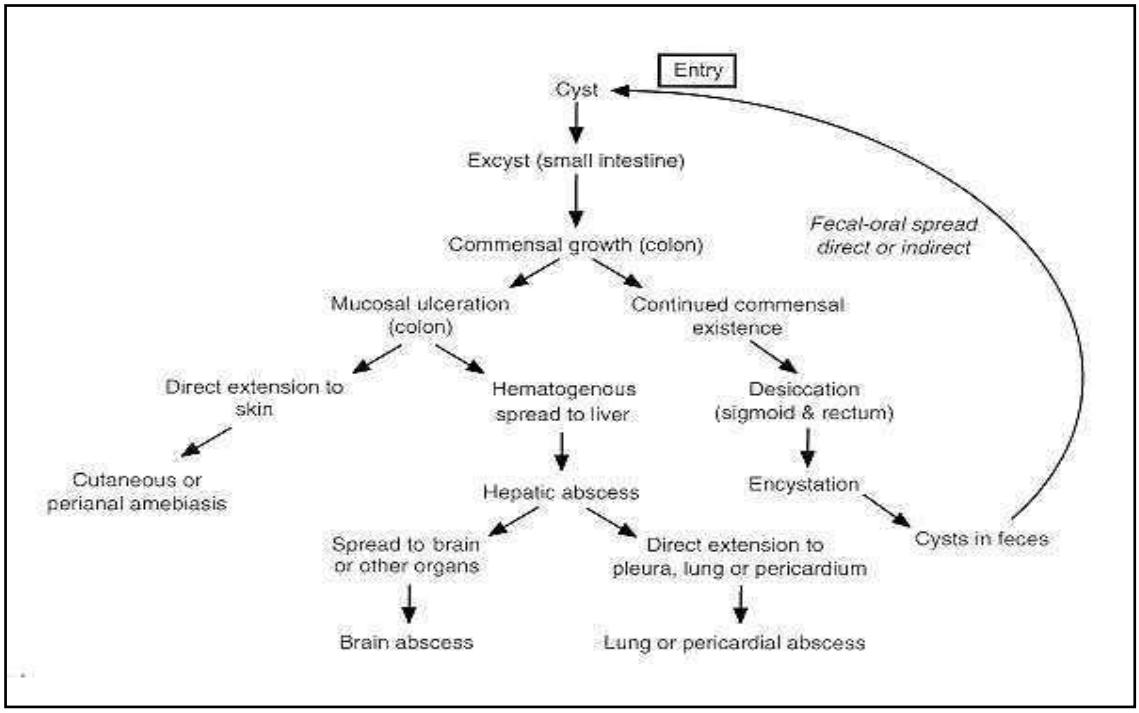


Figure 2.3: Life cycle of *Entamoeba histolytica*

Source: Microbiol Rev 47:84, (1983).

2.8.2.3 Pathogenesis of *Entamoeba histolytica*

The trophozoites mature and reproduce in the host's colon. The parasite may lead a commensally existence on the mucosal surface and in the crypts of the colon. Successful colonization depends on factors such as inoculum size, intestinal motility, transit time, the presence or absence of specific intestinal flora, the host's diet and the ability of the amoeba to adhere to the colonic mucosal cells (Sodeman and William, 1990). The amoeba adherence molecule has been identified as a lectin which can bind to either of two common carbohydrate components of cell membrane, galactose and *N*-acetyl galactoseamine (Gonin and Trudel, 2003; Weber *et al.*, 2008). Binding to colonic mucins blocks adherence to mucosal cells. Depletion of mucus results in binding to the mucosa, an essential step in the development of the disease. The amoeba attacks and kills the host cell. Binding involving the galactose adherence lectin is essential for the cytolytic effect. This cytolytic event is a result of incorporation in the host cell membrane of an amoeba-

produced, pore-forming protein, amoebapore (Sodeman and William, 1990). This protein forms ion channels in lipid cell membranes and results in cell death within minutes of cell contact with the amoeba. Non-pathogenic strains of *E. histolytica* can also produce amoebapore but are much less efficient at its production and the molecule is not exactly similar to that produced by virulent strains (Mahon and Manuselis, 2000; Washington Winn *et al.*, 2006).

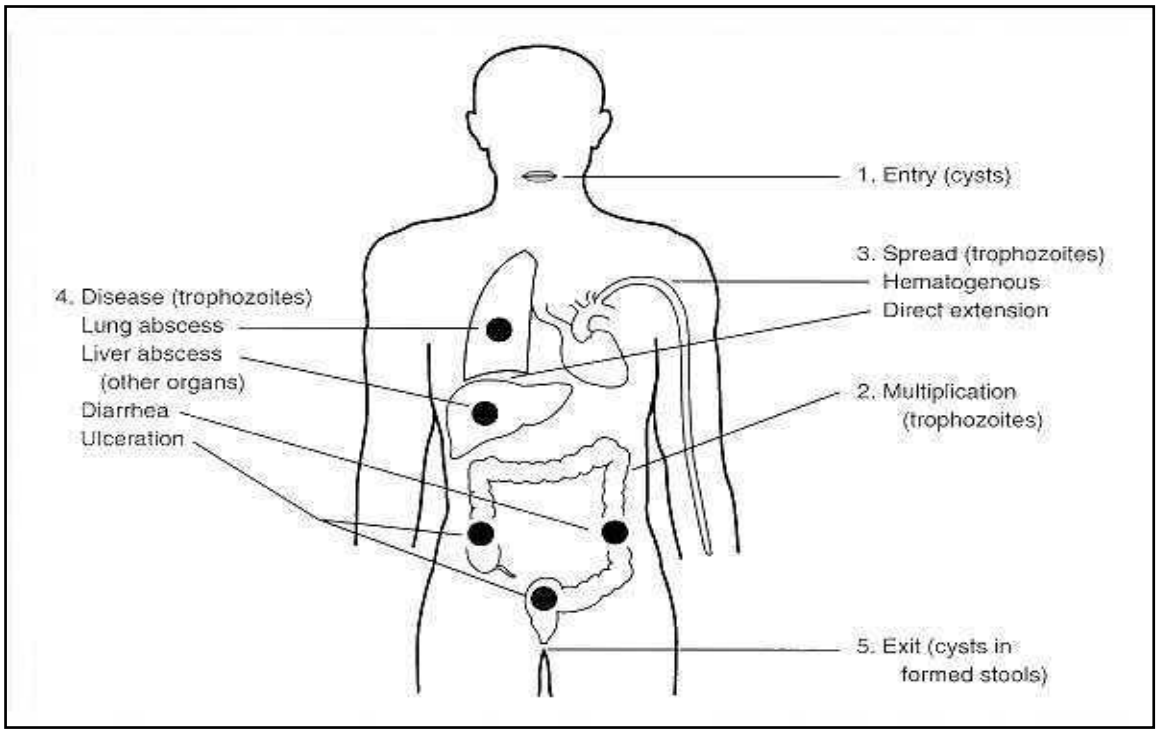


Figure 2.4: Pathogenesis of *Entamoeba histolytica* infection
Source: Microbiol Rev 47:84, (1983).

The initial lesion occurs in the colonic mucosa, most often in the cecum or sigmoid colon. The slow transit of the intestinal contents in these two locations seems an important factor in invasion of the mucosa, both because it affords the amoeba greater mucosal contact time and because it permits changes in the intestinal milieu that may facilitate invasion. The initial superficial ulcer may deepen into the sub mucosa and muscularis to become the characteristic flask-shaped, chronic amoebic ulcer (Guerrant, 1986). Spread may occur by direct extension, by

undermining of the surrounding mucosa until it sloughs, or by penetration that can lead to perforation or fistulous communication to other organs or the skin. If the amoeba gain access to the vascular or lymphatic circulation, metastases may occur first to the liver and then by direct extension or further metastasis to other organs, including the brain (Weber *et al.*, 2008).

Virulent *E histolytica* strains are capable of penetrating intact intestinal mucosa. The infection is not opportunistic and does not require pre-existing mucosal damage (Chacon-Cruz et al., 2007). Numerous proteases have been isolated in *E histolytica*; however, the mechanism of penetration remains unclear (Garfinkel *et al.*, 1989). Metastatic foci present as abscesses with a central zone of lytic necrosis surrounded by a zone of inflammatory cell infiltration (Ravdin, 1986). Metastatic abscesses behave as space-occupying lesions unless they become secondarily infected or rupture (Sodeman and William, 1990).

The clinical presentation of intestinal infections depends on the extent and anatomic location of the ulceration and mucosal damage (Leippe and Muller-Eberhard, 1994). Small, sparse ulcerations may be asymptomatic. As the involved area of the mucosa increase in size and/or in depth, motility disturbances occur, primarily diarrhea with cramping pain. Exudation from the denuded mucosa adds to intestinal content (Petri and Mann, 1993). When the mucosal involvement becomes extensive, diarrhoea is replaced by dysentery, with the passage of exudates, blood and mucus (Enrique Chacon-cruz and Douglas Mitchell, 2007), 2007). Toxic megacolon and perforation are rare complications of extensive involvement (Weber *et al.*, 2008). Systemic signs of infection include fever, rigor and polymorphonuclear leukocytosis (Singh, 1975).

2.8.2.4 Clinical manifestations of *Entamoeba histolytica* infection

About 80-99% of all humans infected with *E. histolytica* are asymptomatic (Gonin and Trudel, 2003). The majority of those with symptoms have the disease limited to the gastrointestinal tract. The average incubation period is 1-4 weeks

(Washington Winn *et al.*, 2006). If mucosal invasion occurs, it may be limited to a few simple superficial erosions or it may progress to total involvement of the colonic mucosa with ulceration (Weber *et al.*, 2008). The clinical manifestations vary with the extent of involvement. Mucosal erosion causes diarrhoea, which increases in severity with size and depth of the area. Symptoms are also affected by the site of the infection. The more distal the lesion in the colon, the greater the likelihood and severity of symptoms; thus small rectal lesions are more likely to be symptomatic than larger cecal lesions. Rectal bleeding is only slightly less common than diarrhoea and is usually, but not invariably, associated with diarrhoea. Such bleeding may be grossly apparent or may be occult and demonstrable only by chemical testing for blood. Urgency, tenesmus, cramping abdominal pain and tenderness may be present (Weber *et al.*, 2008).

The intestinal syndromes caused by *E. histolytica* form a continuum ranging in severity from mild diarrhoea to hemorrhagic dysentery. The span from mild to severe diarrhoea is classified as non-dysentery colitis (Gonin and Trudel, 2003). Amoebic dysentery has a dramatically different clinical presentation. The diarrhoea is replaced by dysenteric stools consisting largely of pus and blood without faeces. There is evidence of systemic toxicity with fever, dehydration, and electrolyte abnormalities. Tenesmus and abdominal tenderness are regular features. This fulminant presentation may occur suddenly or evolve from less severe, pre-existing disease (Guerrant, 1986). Occasionally, and for no apparent reason, colonic infection with *E. histolytica* will evoke a proliferative granulomatous response at an ulcer site. This infectious pseudo-tumor, called an amoeboma, may become the leading point of intussusceptions or may cause intestinal obstruction. This complication is uncommon (Enrique Chacon-cruz and Douglas Mitchell, 2007).

Peritonitis as a result of perforation has been reported in connection with severe amoebic colitis and, much less often, in patients with few or no symptoms (Gonin and Trudel, 2003). Other complications of intestinal amoebiasis include

colocutaneous fistula, perianal ulceration, urogenital infection, colonic stricture, intussusceptions and haemorrhage (Mahon and Manuselis, 2000). Most of these complications are uncommon and therefore may prove difficult to diagnose. The term post-amoebic colitis is used for nonspecific colitis following a bout of severe acute amoebic colitis. In such cases, the colon is free of parasites and the clinical findings resemble those of chronic ulcerative colitis (Mahon and Manuselis, 2000) 2000).

Extra intestinal amoebiasis begins with hepatic involvement (Qvarnstrom *et al.*, 2005). Many patients with acute intestinal infection also have hepatomegaly, but in these cases amoebae are not demonstrable in the liver and the pathogenesis of this hepatomegaly is not clear (Adams and MacLeod, 1977). A focal amoebic abscess in the liver represents metastasis from intestinal infection. This may be within days after acute bout of dysentery or it may be delayed by months or years (Adams and MacLeod, 1977). Symptomatic intestinal infection need not be present. The abscess appears as a slowly enlarging liver mass. Often the patient will have right upper quadrant pain, which may be referred to the right shoulder. If the abscess is located in a palpable portion of the liver, the area will be tender. Occasionally the enlarging abscess presses on the common bile duct and causes jaundice (Washington Winn *et al.*, 2006). If located under the dome of the diaphragm, the abscess may cause elevation of the dome of the diaphragm which presses on the right lung base, causing atelectasis and physical findings of consolidation. As the abscess nears the diaphragm the inflammation may stimulate pleural effusion (Washington Winn *et al.*, 2006). Pleural, pulmonary, and pericardial infection occurs as a result of direct extension from the liver. Lung involvement is far more common than pericardial infection. Infection metastatic from the liver can involve other viscera or can give rise to a brain abscess. However, these complications are uncommon (Strachan *et al.*, 1988).

2.8.2.5 Host defenses to *Entamoeba histolytica* infection

The gastric acid "barrier" and the steady movement of food through the intestine are nonspecific defense mechanisms invoked to explain observation that large inocula are required to produce consistent infection in animals and the pathologic observation that few lesions are found in the small intestine, a zone of rapid transit (Sodeman and William, 1990). A role for colonic mucins in protection and depletion of these mucins in infection has been suggested (Enrique Chacon-cruz and Douglas Mitchell, 2007). Usually amoebae alone stimulate little or no direct cellular response. Primary intestinal lesions elicit little reaction until secondary bacterial infection occurs. Amoebic abscesses similarly elicit only a mild leukocytic response, which may be largely a response to the host cellular debris in the abscess (Sodeman and William, 1990). Amoebae are antigenic and stimulate an antibody response and cellular sensitivity. The occurrence of progressive and/or recurrent infection in the face of established immune sensitivity suggests that the host immune response is relatively ineffective against established infections (Knobloch and Mannweiler, 1983).

2.9 Diagnosis of intestinal protozoa parasites

Intestinal protozoa are diagnosed by identifying cysts or trophozoites in fecal specimens or histologically by visualizing cysts in biopsy specimens or secretions of intestinal mucosa (Sheehan *et al.*, 1979). Serological diagnostic methods have been developed such as the Immunofluorescence (IF), Enzyme-linked immunosorbent assay (ELISA), culture and subsequent differentiation by isoenzyme analysis and the Polymerase Chain Reaction (PCR) (Nunez *et al.*, 2001). The conventional direct wet mount preparation for microscopy to identify motile trophozoites and a formalin-ethyl acetate concentration step to identify cysts and (Qvarnstrom *et al.*, 2005). When appropriately conducted with or without iodine stain, the conventional wet mount establishes the diagnosis of *Giardia lamblia* in up to 70-85% of cases after two stool examinations (Sodeman and William, 1990). The sensitivity of the acid-fast stain for oocysts of *Cryptosporidium* in the direct

examination of stools is approximately 30% after one sample examination (Sodeman and William, 1990). Haque *et al.*, (1998) found out that not only is microscopy unable to differentiate *E. histolytica* from *E. dispar*; it is at best only 60% sensitive and can be confounded by false-positive results due to misidentification of macrophages and nonpathogenic species. The conclusion drawn from their study was that of all children with diarrhoea diagnosed with amoebiasis by microscopy, only 40% were proven to have *E. histolytica* infection when other methods (antigen detection and culture-isoenzyme analysis) were used.

In the diagnosis of amoebic liver abscess and extra intestinal illnesses, serological tests are the diagnostic methods used (Sodeman and William, 1990). An indirect hemagglutination assay titre higher than 12 or ELISA titre higher than 40U is more than 99% and is more than 95% specific (Sodeman and William, 1990). van Doorn *et al*. (2005) found the specificities of the ELISA, Dipstick (Schleicher and Schuell Bioscience, Dassel, Germany) and Latex agglutination test (LAT; Laboratories Fumouze Diagnostics, Levallois-Perret, France) for the detection of anti-*Entamoeba histolytica* antibodies in serum of 238 patients and sera from healthy blood donors to be 97.1%, 98.1%, and 99.5%, respectively. In a study conducted by Fotedar *et al*. (2007) in Australia, the PCR assay showed a sensitivity of 81% compared to microscopy in the detection of *Entamoeba histolytica*. A PCR sensitivity of 71.7% was reported by Roy *et al.,* (2005), when they used unfixed, frozen stool samples for the detection of *Entamoeba histolytica*. Hiatt *et al.,* (1995) reported the sensitivity of microscopy for the detection of *Entamoeba* by examination of a single fecal sample to be about 70% (Blessmann *et al.*, 2002).

ELISA has been used for the detection of *Giardia lamblia* antigen in stool samples. Sensitivities of 92-98% and specificities of 87-100% have been reported (Sodeman 1990). Janoff *et al.,* (1989) compared microscopy with counterimmunoelectrophoresis (CIE) and ELISA in the diagnosis of *Giardia lamblia* and found sensitivities of 88% and 94% and specificities of 97% and 95% respectively. The positive predictive values were found to be 86% and 76% whiles

negative predictive values were 98% and 97% respectively. Garcia and Shimizu (1997) reported 100% sensitivity and specificity in the detection of *Giardia lamblia* cyst and *Cryptosporidium* oocyst in faecal specimen using the Merifluor direct immunofluorescence (Meridian Diagnostics) detection systems. Sensitivities of 98-99% and a specificity of 100% were obtained by Garcia and Shimiz in the detection of *Cryptosporidium* oocysts when they used the ProSpecT antigen detection test kit (Alexon).

Although the introduction of these assays have brought improvement in the diagnosis of protozoan parasites, Gonin and Trudel (2003) in their study in Quebec showed that the TechLab ELISAs (TechLab, Inc., Blacksburg, VA) lacked sensitivity for differential diagnosis of *E. histolytica/dispar* complex. They indicated that PCR analysis of frozen stool samples provides reliable results useful as a reference test for sensitive differentiation of species of *E. histolytica* and *E. dispar*. The development of a multiplex real-time PCR for the simultaneous detection of *E. histolytica*, *G. lamblia*, and *C. parvum* in fresh stool samples is apt for the detection of these pathogenic protozoa. With an internal control to determine the efficiency and inhibition in the sample, the multiplex real-time PCR assay has achieved a 100% specificity and sensitivity (Blessmann *et al.*, 2002; Verweij *et al.*, 2004).

Chapter 3

MATERIALS AND METHODS

3.1 Study design

A hospital-based longitudinal study was conducted at the Agogo Presbyterian Hospital in the Ashanti Akyem North District, Ashanti Region. All children up to 13 years attending the Child Welfare Clinic from June, 2007 to May, 2008 were recruited after fulfilling the inclusion criteria.

3.2 Study area

The Asante Akyem North District is one of the 21 Districts in the Ashanti Region. The District is located in the eastern part of Ashanti Region. It covers a land area of 1,160 sq. km with an estimated population of 142,434 (projection from 2000 Population Census). Over 40% of the population is under 15 years of age and over 50% is under 20years. Population aged 65 and above consists of 6.4% of the total population (www.asanteakimnorth.ghanadistricts.gov.gh). The vegetation of the study area is mainly rain forest. The climate is tropical. The temperature variation is between 20 and 36 with monthly rainfall varying from 2.0mm in February to 400mm in July. The major occupation of the people is subsistence farming, animal husbandry and forestry. The sub-districts are Konongo-Odumasi, Agogo, Juansa, Dwease-Praaso and Amanteman. Reports obtained from the Regional Health Directorate, Ashanti Region indicated that, the Agogo Presbyterian Hospital which is the major hospital in the district recorded a total of 54,174 outpatient attendance in the 2007 fiscal year. The top 10 cases reported were acute eye disease (17%), Malaria (15.1%), Upper respiratory tract infections (5.9%), Diarrhoea (3.2%), Skin disease and Ulcer (2.1), Cataract (1.8%), acute urinary tract infection (1.8%), gynaecological conditions (1.7%), acute ear infections (1.3%), vaginal discharge (1.2%) and all other conditions (48.0%) (The major sources of water in the district

include pipe borne, borehole, stream and well. Environmental Health and Sanitation issues are major problems facing the district.

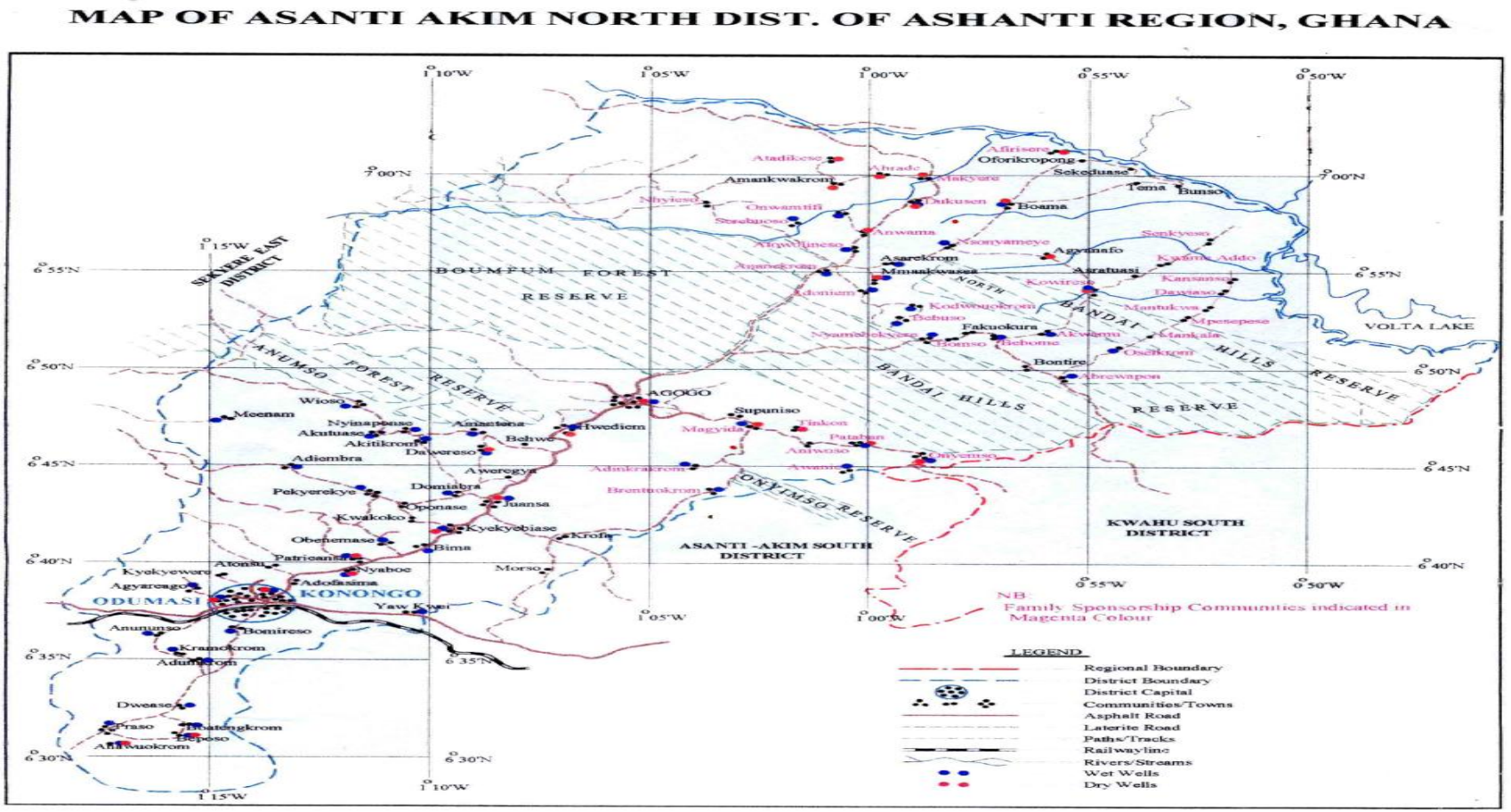


Figure 3.1: Ashanti Akyem North District of Ashanti Region.

Source: Ghana Survey Department

3.3 Study site

The Agogo Presbyterian Hospital is a major hospital serving the Ashanti-Akyem North District and other parts of the Ashanti region. It is the oldest mission hospital in Ghana established in 1931. The hospital's coverage population is approximately 70,000 people. The Child Welfare Clinic of the hospital offers outpatient services to children from birth up to 13 years. The clinic is managed by a paediatric team of doctors: 1 paediatrician, 2 residents in paediatrics, 2 medical officers and 2 house officers, 2 nurses and 3 ward and health aids supported by 3 administrative staff.

The laboratory department of the hospital offers diagnostic as well as research services. The department is fully equipped with two modern automated blood culture incubators (BACTEC 9050 BD Diagnostics Sparks Massachusetts, USA), a carbon dioxide incubator, 2 safety cabinets for bacteriological culture and sensitivity testing, a Sysmex KX21N for Heamatology, one Selectra Junior and two Vita Lab FlexorE for Biochemistry. There are 6 Light microscopes, scientific fridges and freezers as well as Centrifuges and Water baths. The laboratory is in collaboration with the Kumasi Centre for Collaborative Research (KCCR) of the Kwame Nkrumah University of Science and Technology (KNUST) where facilities (PCR, RT-PCR, Light Cycler, -80^{o}C freezers) for molecular biology works are available. Laboratory tests are carried out from approved standard Operating Procedures (SOP) and every activity undertaken in the laboratory is well documented. The department participates in the quarterly External Quality Assessment program of the United Kingdom (UK) National External Quality Assessment Scheme (UK NEQAS) in Haematology and Clinical Chemistry and the National Institute of Communicable Diseases/National Health Laboratories (NICD/NHL) in Clinical Microbiology and Parasitology from South Africa.

The study was approved by the Committee on Human Research, Publications and Ethics (CHRPE), School of Medical Sciences, Kwame Nkrumah University of

Science & Technology (KNUST), Kumasi. This study was part of a major study on Neglected Infectious Diseases in children in the district.

3.4 Study population

All Children of up to 13 years old and permanently residing in the Ashanti Akyem North District who attend the Child Welfare Clinic of the Agogo Presbyterian hospital were included in this study if they fulfilled the inclusion criteria.

3.5 Inclusion criteria

3.5.1 Symptomatic children

All children with diarrhoea and or vomiting within the last 3 days, heamoglobin levels <8g/dl (all anaemic cases besides haemolytic or heamorrhagic anaemia) and urticaria. Also children with malnutrition (weight/height Z-score ≤ -2, Deficiency of growth: weight/age Z-score ≤ -2) were enrolled into the study.

3.5.2 Asymptomatic children (Control)

All children who attended the clinic for their normal checkups or with ailments other than what are stated in 3.5.1 above were enrolled. The first 7 of these children seen each day were used as controls.

Signed informed consent was obtained if the potential parent demonstrated understanding of the study and was willing to enroll his/her child. In the case of an illiterate parent, a left thumbprint was obtained on the consent forms and a separate Witness Consent form was signed by a literate witness who had observed the consent processes. The interview was done in Twi which is the local language in the district.

3.6 Exclusion criteria

Children who needed emergency treatment and those whose parents/guardians would not give their consent.

3.7 Sampling

Labeled sterile containers with a collecting spoon were provided to all the children and/or their mothers who fulfilled the inclusion criteria for the provision of stool samples. The date and time the sample was received was noted in a separate book.

3.7.1 Sampling methods

The decision concerning evaluation of clinical symptoms was made by the paediatrician at the child welfare clinic according to the enrolment criteria.

3.8 Laboratory investigations

3.8.1 Sample processing

One stool sample was collected from each child. The Fresh stool samples were transported to the laboratory immediately for analysis. Stool samples (0.2g) were kept in a labeled 2.0ml Eppendorf tubes and frozen at -20^{o}C without preservative for molecular analysis. The remaining portions of the stools were preserved in Sodium-acetate acetic acid formalin solution (SAF) (appendix 6) for microscopy and the formol-ether concentration method (appendix 7). The concentrate (sediments) was divided into two portions. One portion in a 15ml Falcon tube was stained with Lugol's iodine. Smear preparation of the other portion on clean dry 76mm x 26mm microscopy slide was stained with the modified Kinyoun stain (appendix 8).

3.8.2 Microscopy

The Lugol's iodine stained concentrate was well mixed using a sterile Pasteur pipette. A drop was placed on a labeled clean dry 76mm x 26mm slide and covered with a 20mm x 20mm cover slide. The preparation was examined with a Zeiss light microscope (Aziostar plus, Carl Zeiss Microimaging, Germany) using the low power (x10) and the high power (x40) objectives for helminth larvae and ova identification. The 40x and 100x objectives were used for searching and identification respectively of cysts and the trophozoites of protozoa. The modified Kinyoun stained slides were examined with the 40x and then 100x objectives for

Cryptosporidium parvum oocysts. The following features confirmed the identification of the pathogenic protozoa: (1) Oval shaped, thick wall cyst size of 8-19µm in diameter with 2-4 nuclei at one end and median bodies were identified as Giardia lamblia cyst. The trophozoites possess four pairs of flagella, two nuclei, without undulating membrane (axostyle) about 10-20µm in diameter.

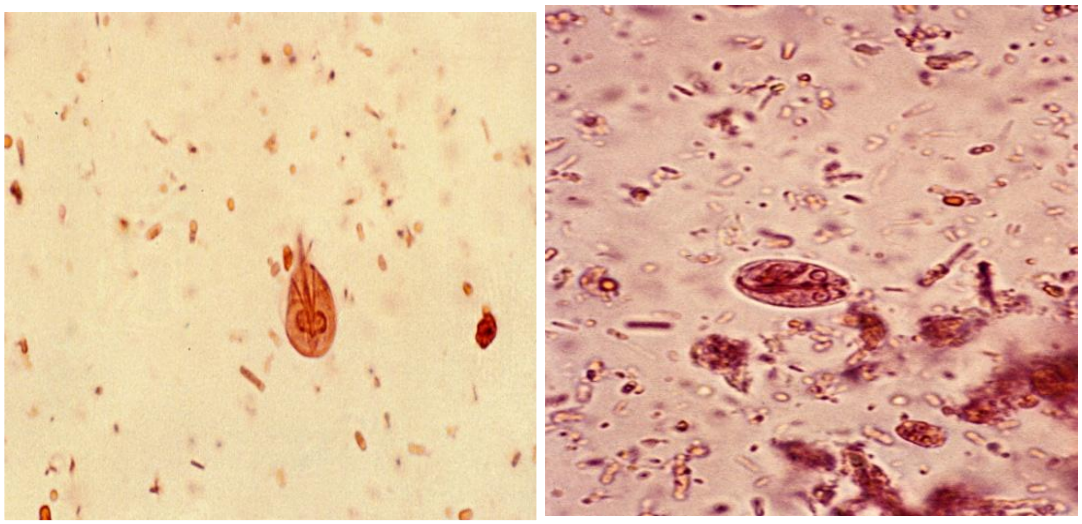


Plate 3.1: Lugol's iodine stained *Giardia lamblia* trophozoite and cyst
Source: Colour Atlas and Textbook of Diagnostic Microbiology

(2) Cysts of size 10-20µm, peripheral nuclear chromatin, 1-4 nuclei, glycogen mass and chromatin bodies identify *Entamoeba histolytica* cysts. The size of the trophozoites ranges from 15-60µm, they possess one nucleus with fine and regularly arranged peripheral nuclear chromatin. The finely granulated cytoplasm may contain red cells.

(3) The oocyst of *Cryptosporidium parvum* were stained pink to red (Acid fast) with black granules. Their size ranges from 4-6µm (WHO 1994).

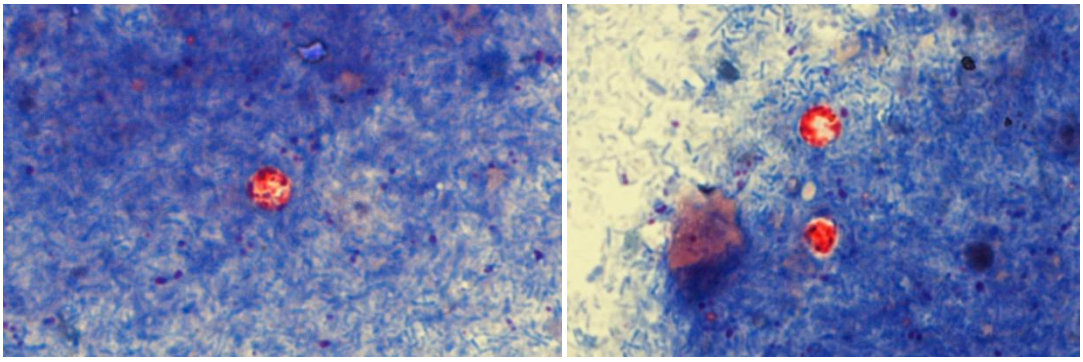


Plate 3.2: Kinyoun stained *Cryptosporidium parvum* oocyst
Source: Colour Atlas and Textbook of Diagnostic Microbiology

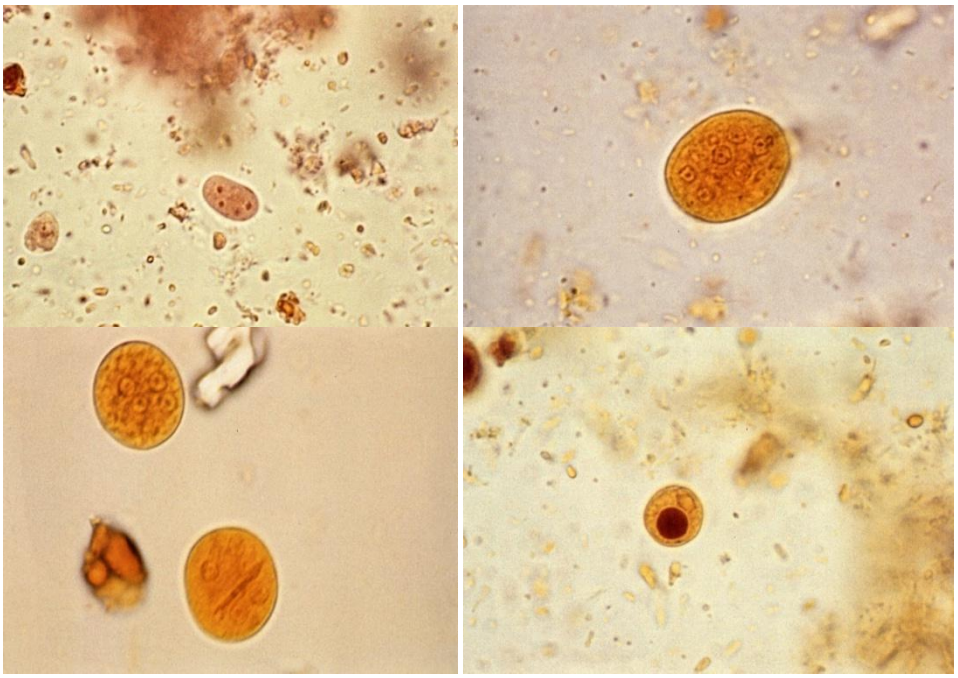


Plate 3.3: Lugol's iodine stained Cysts of *Entamoeba* species
Source: Colour Atlas and Textbook of Diagnostic Microbiology.

3.8.3 Polymerase chain reaction
3.8.3.1 DNA extraction

DNAs of up to 20kb of *Giardia lamblia, Cryptosporidium parvum, Entamoeba histolytica, Entamoeba dispar* in the unpreserved stool samples were extracted and purified using the QIAamp DNA Stool mini kit (Qiagen, Hilden, Germany). The extraction was done according to the manufacturer's protocol (appendix 9).

3.8.3.2 Real Time PCR

Real time PCR assay was performed on DNA eluates from all the stool samples using the Corbett Rotor Gene 6000 (Corbett life sciences, Australia) using fluorescence-labeled detection probes and primers amplifying a 310-bp fragment from the high-copy-number, ribosomal DNA-containing *Amoeba, Giardia* and *Cryptosporidium* episomes. The primers used were specific for the respective parasite species.

Entamoeba histolytica Primer:

Ehd-239F (Oligo 1164) 5'-ATTGTCGTGGCATCCTAACTCA-3'
Ehd-88R (Oligo 1165) 5'-GCGGACGGCTCATTATAACA-3'
histolytica-96T (Oligo 1168) 5'-Joe-TCATTGAATGAATTGGCCATTT-BHQ1-3'

Giardia lamblia Primer:

Giardia F (Olio 1006) 5'-GACGGCTCAGGACAACGGTT-3'
Giardia R (Olio 1007) 5'-TTGCCAGCGGTGTCCG-3'
Giardia T (Oligo 1248) 5'-Fam-CCCGCGGCGGTCCCTGCTAG-TAMRA-3'

Cryptosporidium parvum Primer:

Crypto F (Oligo 1054) 5'-CGCTTCTCTAGCCTTTCATGA-3'
Crypto R (Oligo 1055) 5'-CTTCACGTGTGTTTGCCAAT-3'
Crypto T (Oligo 1196)5'-RoxCCAATCACAGAATCATCAGAATCGACTGGTATC-BHQ2-3'

For One single reaction, the reaction mixture contains 12.5 µl HotStarTaq Mastermix 2x (QIAGEN, Valencia, and Calif.), 3.5 µl MgCl2 with a concentration of 25 mM and 4.25µl Distilled water.

The primer volumes and concentrations were:

Primer volume	**Primer conc.**	**Final conc.**
0.25 µl Ehd-F	12.5 pmol/µl	3.125 pmol
0.25 µl Ehd-R	12.5 pmol/µl	3.125 pmol
0.25 µl histolytica T	3.5 pmol/µl	0.875 pmol
0.25 µl Giardia F	12.5 pmol/µl	3.125 pmol
0.25 µl Giardia R	12.5 pmol/µl	3.125 pmol
0.25 µl Giardia T	5.0 pmol/µl	125 pmol

0.25 µl Crypto F	50.0 pmol/µl	12.5 pmol
0.25 µl Crypto R	50.0 pmol/µl	12.5 pmol
0.25 µl Crypto T	17.5 pmol/µl	4.375 pmol
2.5 µl DNA		
Total amount: 25.0 µl		

All primers were obtained from MWG diagnostics Inc., (Netherland). The primers were kept at 4°C.

Reaction conditions were chosen according to a standard Rotor Gene protocol at the KCCR laboratory and were 15 min at 95°C, followed by 40 cycles of 15 sec at 95°C, 30sec at 64°C and 30 sec at 72°C and a final hold at 40°C for 30 sec. A positive DNA-sample is recognized by an exponential increasing florescence signal above the threshold set at 35. The reaction also included an internal control to determine efficiency of the PCR and detect inhibition in the sample.

3.8.4 Statistical analysis

All data collected were entered into a fourth dimensional computer database. The data were analyzed using the *Stata*/SE9.0 statistical software (Stata Corporation, Texas USA). Results were evaluated by the chi-square and Fisher exact tests as indicated for the analysis of socioeconomic, demographic and clinical characteristics of study children. The prevalence of Protozoa and helminth infections, association of age and the risk of protozoa infection and the association of diarrhoea and/or vomiting with protozoa infection were determined. A *P value* of less than 0.05 was considered significant. The performance characteristics (sensitivity, specificity, positive and negative predictive values) of microscopy and the RT PCR techniques were compared to an expanded gold standard (microscopy and PCR positives, PCR only and microscopy only positives). The measure of agreement between the microscopy and the RT PCR techniques was determined using the kappa statistics.

Chapter 4

RESULTS

4.1 Enrollment outcome of the study children

During the study period, a total of 1506 children were enrolled. They were made up of 855 (56.77%) symptomatic and 651 (43.23%) asymptomatic children. Out of the total number, 1373 (91.17%) provided their stool samples for analysis. However, 8.83% (133) did not provide specimens because they were either withdrawn or were lost to follow up. The monthly enrollment of both asymptomatic and symptomatic children is shown in **Figure 4.1**. The highest monthly enrollment was in October 2007 whiles the least was in March 2008.

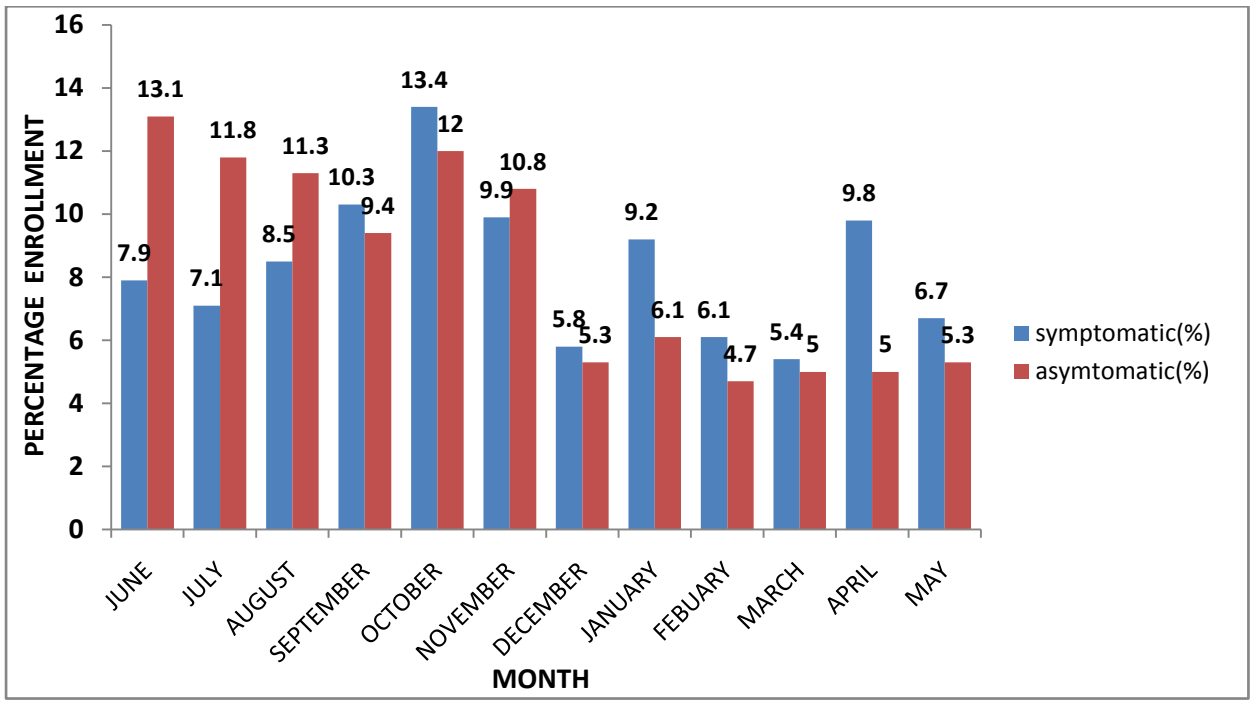


Figure 4.1: Monthly enrollment of study children (June2007 to May 2008)

4.2 Socioeconomic characteristics of study children

More than sixty percent (60.52%) of the children who were able to provide stool samples were symptomatic. Of these symptomatic children, over forty percent (47.1%) reside in the Agogo sub district (**Table 4.1**). The majority of these children are of Akan

ethnicity. The source of water supply for majority of the children was an outside stand pipe while the Kumasi Ventilated Improved Pit (KVIP) was the main toilet facility in the district (**Table 4.1**).

Table 4.1: Socioeconomic characteristics of study children

	Asymptomatic N=523	**Symptomatic N=802**	**Total N=1,325**
RESIDENCE	n (%)	n (%)	n (%)
Agogo	398(76.1)	378(47.1)	776(58.6)
Domeabra	32(6.2)	19(2.4)	51(3.9)
Hwidiem	46(8.8)	38(4.7)	84(6.3)
Juansa	19(3.6)	27(3.4)	46(3.5)
Konongo	7(1.3)	165(20.6)	172(13.0)
Others	21(4.0)	173(21.6)	194(14.6)
Ethnicity			
Akan	442(84.5)	669(83.4)	1,111(83.9)
Northern	65(12.4)	91(11.4)	156(11.8)
Others	16(3.1)	42(5.2)	58(4.4)
House type			
Cement	59(11.3)	86(10.7)	145(10.9)
Mud	4(0.8)	4(0.5)	8(0.6)
Electricity			
No	11(2.1)	22(2.7)	33(2.5)
Yes	51(9.8)	68(8.5)	119(9.0)
Water source			
Inside tap	18(3.4)	24(3.0)	42(3.3)
Stand pipe	29(5.5)	51(6.4)	80(6.0)
Well	12(2.3)	12(1.5)	24(1.8)
Toilet facility			
KVIP	36(6.9)	40(5.0)	76(5.7)
Water closet	5(1.0)	14(1.8)	19(1.4)

4.3 Demographic and clinical characteristics of study children

The ages of study children ranged from 3 months to 13 years. Majority of the children were under 5 years. The average age was 3.25 years. The mean ages of the symptomatic and asymptomatic children were 2.40 and 4.56 years respectively. A Significant number of the symptomatic recruited children were younger than the asymptomatic children (p<0.001) (**Table 4.2**). There were 46% and 54% females and males respectively. The female to male ratio was 1:1.7. There was no significant association between sex and whether the recruited children were symptomatic or asymptomatic (p=0.180) (**Table 4.2**).

Table 4.2: Demographic and clinical characteristics of study children

Parameter	Total n (%)	Symptomatic n (%)	Asymptomatic n (%)	P value
Male	742(54.04)	437(52.59)	305(56.27)	
Female	631(45.96)	394(47.41)	237(43.73)	0.180
Mean Age(year)	3.25 ±0.09	2.40 ± 0.10	4.56 ± 0.15	<0.001
Nutritional condition				
Weight/Age Z-score <=-2	119(12.18)	119(17.45)	0(0.00)	<0.001
Weight/Height Z-score <=-2	107(10.95)	107(15.69)	0(0.00)	<0.001
Malnutrition	37(2.70)	37(4.45)	0(0.00)	<0.001
Reported symptoms				
Diarrhoea	511(37.22)	511(61.49)	0(0.00)	<0.001
Mean Diarrhoea duration in days		3.99 ± 0.24		
Mean diarrhoea frequency/day		4.20 ± 0.06		
Vomiting	474(34.55)	474(57.11)	0(0.00)	<0.001
Mean vomiting duration in days		2.12 ± 0.12		
Mean vomiting frequency/day		2.27 ± 0.10		
Dehydration	26(1.9)	25(3.03)	1(0.19)	<0.001
Cough	656(47.85)	377(45.42)	279(5157)	0.026
Fever	1079(78.59)	633(76.17)	446(82.29)	0.007
Skin rash	148(10.82)	79(9.54)	69(12.78)	0.060
Stool consistency				
Firm	19(5.18)	5(1.92)	14(13.08)	<0.001
Loose	287(78.20)	194(74.62)	93(86.92)	0.009
Mucoid	94(25.61)	89(34.23)	5(4.67)	<0.001
Watery	58(15.80)	58(15.80)	0(0.00)	<0.001
Blood stained	7(1.91)	7(1.91)	0(0.00)	0.087

A significant number of the symptomatic children were stunted, underweight and wasted (p <0.0001) (**Table 4.2**). Gastrointestinal symptoms reported included diarrhoea, vomiting and dehydration. The average duration and frequency of diarrhoea were 3.9 days and 4.2 times a day, whilst the average duration and frequency of vomiting was 2.1 days and 2.7 times a day respectively (**Table 4.2**). A significant number (76.17%) of the children enrolled over the study period reported with fever, temperatures >37^0C (p= 0.007) (**Table 4.2**). The consistency of the stool samples submitted by the symptomatic children ranged from loose, watery (p <0.0001) mucoid, (p <0.0001), bloodstained and firm (p <0.0001) (**Table 4.2)**.

4.4 Protozoa and helminths infections in children by microscopy

The overall prevalence of helminths found in the study children was 2.4%. Hookworm, *Hymenolepis nana* ova, *Ascaris lumbricoides* ova and *Strongyloides stercoralis* larvae were the helminths found during microscopic examination in the children. Generally helminths infection in this study was low compared to the protozoa infection in the district (**Table 4.3**).

Table 4.3: Infection rate of helminths in study children by microscopy

Organisms	**Total N=1242**	**Symptomatic N =742**	**Asymptomatic N =500**	***P value***
	n (%)	n (%)	n (%)	
Ascaris lumbricoides	2(0.16)	1(0.13)	1(0.20)	0.779
Hookworm	7(0.56)	4(0.54)	3(0.60)	0.888
Strongyloides stercolaris	12(0.97)	7(0.94)	5(1.00)	0.920
Hymenolepis nana	3(0.24)	1(0.13)	2(0.40)	0.350
Hymenolepis diminuta	2(0.16)	1(0.3)	1(0.20)	0.779
Metagonimus species	7(0.56)	5(0.67)	2(0.40)	0.527

The prevalence of protozoa parasites detected by microscopy was 37.7%. *Giardia lamblia* (15.97%) was the most frequently detected pathogenic protozoan in the stool samples of symptomatic (12.96%) as well as asymptomatic (20.54 %) children in the study (**Table 4.4**). The prevalence of non-pathogenic protozoa, *Entamoeba coli,*

Entamoeba hartmani, Endolimax nana, Chilomastix mesnelix and *Blastocystis* detected were 7.41%, 1.73%, 4.53%, 4.03% and 14.16% respectively (**Table 4.4**).

Table 4.4: Infection rate of protozoa in study children by microscopy

Organisms	**Total N=1215**	**Symptomatic N =733**	**Asymptomatic N =482**	***P value***
	n (%)	n (%)	n (%)	
Entamoeba histolytica/dispar	9(0.74)	3(0.41)	6(1.24)	0.097
Giardia lamblia	194(15.97)	95(12.96)	99(20.54)	<0.001
Cryptosporidium species	44(4.91)	34(4.92)	10(4.85)	0.969
Isospora belli	2(0.22)	2(0.29)	0(0)	0.439
Entamoeba coli	90(7.41)	29(3.96)	61(12.66)	<0.001
Entamoeba hartmani	21(1.73)	5(0.68)	16(3.32)	0.001
Endolimax nana	55(4.53)	13(1.77)	42(8.71)	<0.001
Chilomastix mesnili	49(4.03)	19(2.59)	30(6.22)	0.002
Blastocystis hominis	172(14.16)	74(10.10)	98(20.33)	<0.001

4.5 Prevalence of protozoa in study children by PCR

The overall prevalence of the three pathogenic protozoa in the study children as detected by PCR was 40.2%. The rate of *Giardia lamblia* infection (34.77%) increased significantly (p <0.0001) with the use of molecular assay compared to microscopy (**Table 4.5**). However the prevalence of *Cryptosporidium parvum* in both microscopy and PCR assay was comparable (4.91% vs. 4.37%). Differentiation of the *Entamoeba* species by PCR assay indicated that only 0.07% were *Entamoeba histolytica* which was detected in only one sub-district (Agogo). Meanwhile, *Entamoeba dispar* (6.4%) was detected in the entire district (**Table 4.5**).

Table 4.5: Prevalence of protozoa in study children by PCR

Organisms	Total N=1372	Symptomatic N (831)	Asymptomatic N (541)	*P value*
	n (%)	n (%)	n (%)	
Cryptosporidium parvum	60(4.37)	47(5.66)	13(2.4)	0.004
Giardia lamblia	477(34.77)	259(31.17)	218(40.30)	0.001
Entamoeba histolytica	1(0.07)	0(0.00)	1(0.18)	0.153

4.6 Concentration of DNA by RT-PCR

More than 80% of *Giardia lamblia* DNA were detected below the threshold cycle (Ct) value of 23 while only 16% were found above Ct 32. In contrast, no *Cryptosporidium parvum* DNA was detected below Ct 23. More *Cryptosporidium parvum* DNA (91%) were found between 24 and 31 ct values and 21% above Ct >32 (**Figure 4.2).**

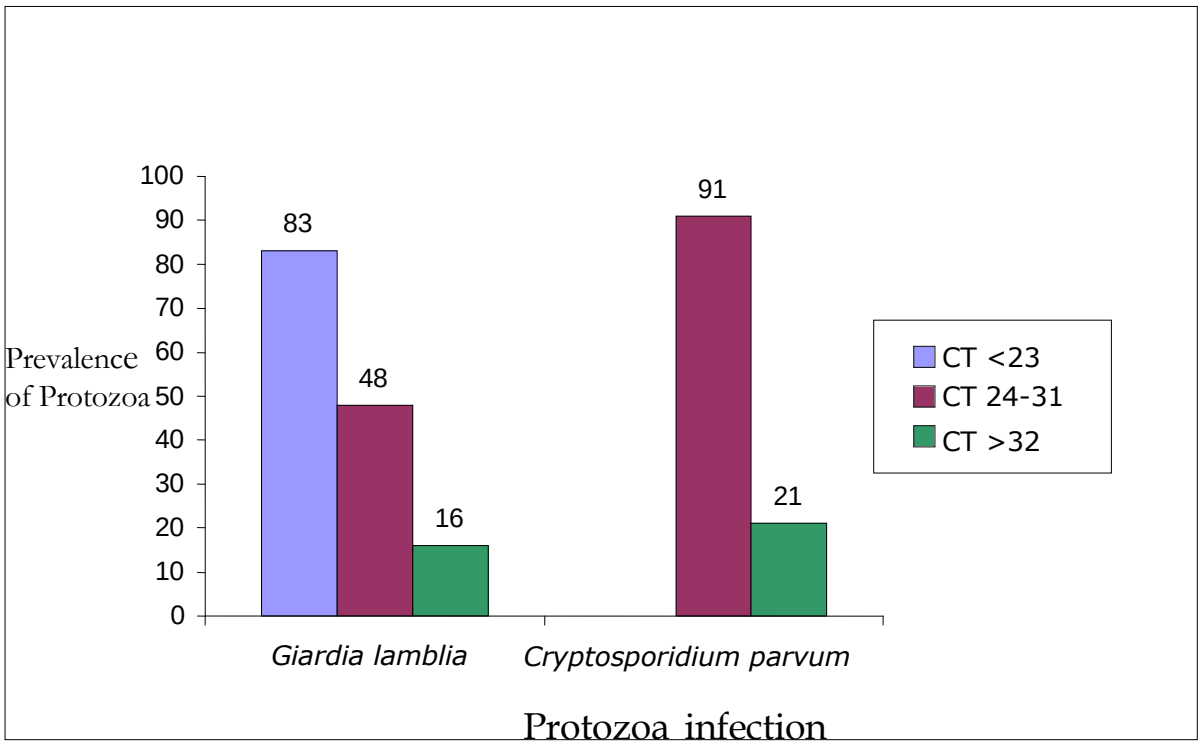


Figure 4.2: PCR Ct values with protozoa infection concentration

4.7 *Cryptosporidium parvum* and *Giardia lamblia* distributions in the sub districts by PCR

Giardia lamblia was the most frequently detected protozoa parasite in stool samples of children from the entire district with an overall prevalence of 34.77 % **(Table 4.5).** Agogo recorded the highest *Giardia lamblia* infection. Out of the 452 symptomatic and 442 asymptomatic children, 162 (56.8%) and 187 (42.3%) were positive respectively in the Agogo sub-district (**Table 4.6**). Juansa recorded the lowest symptomatic (1.4%) and asymptomatic (0.9%) cases of *Giardia lamblia* infection during the study period. No child from Juansa and Odumasi sub districts was found to be infected with *Cryptosporidium parvum* during the study (**Table 4.6**).

Table **4.6**: Prevalence of *Cryptosporidium parvum* and *Giardia lamblia* by PCR

		Symptomatic			**Asymptomatic**	
		Giardia lamblia	***Cryptosporidium Parvum***		***Giardia lamblia***	***Cryptosporidium Parvum***
Residence	N	n (%)	n (%)	N	n (%)	n (%)
Agogo	452	162(56.8)	21(41.2)	442	187(42.3)	11(64.7)
Domeabra	24	7(2.5)	2(3.9)	35	8(3.4)	1(5.88)
Hwidiem	44	13(4.6)	4(7.8)	51	19(8.2)	4(23.5)
Juansa	32	4(1.4)	0	20	2(0.9)	0
Konongo	181	35(12.3)	10(19.6)	16	6(2.6)	0
Others*	200	16(21.4)	14(27.5)	30	11(4.7)	1(5.88)

*Smaller towns within the district

4.8 Distribution of protozoa infection by age using PCR assay

The infection of *Giardia lamblia* in both asymptomatic and symptomatic children was highest among the 13-36 months old age group. There was a significant difference between asymptomatic and symptomatic infection among the 13-24 months age groups (p =0.001) (**Table 4.7**).

Table 4.7: Age distribution and infection rate of *Giardia lamblia* in study children by PCR

Age(month)	Total n (%)	Symptomatic n (%)	Asymptomatic n (%)	*P* value
≦12	36(12.04)	33(13.25)	3(6.00)	0.162
13-24	126(41.58)	76(35.35)	50(56.82)	0.001
25-36	85(50.60)	52(48.60)	33(54,10)	0.493
37-48	57(47.50)	27(40.91)	30(55.56)	0.111
49-60	35(40.23)	19(43.18)	16(37.21)	0.570
≥60	138(34.94)	52(34.67)	86(35.10)	0.930

Cryptosporidium infection was commonly detected in children aged 13 - 24 months. Infection rates of *Cryptosporidium* decreased with increasing age (**Table 4.8**). Symptomatic children with *Cryptosporidium parvum* were predominant among the <12-24 months age groups.

Table 4.8: Age distribution and infection rate of *Cryptosporidium parvum* in study children by PCR

Age(month)	Total n (%)	Symptomatic n (%)	Asymptomatic n (%)	P value
≦12	17(5.69)	17(6.89)	0(0.00)	<0.001
13-24	26(8.58)	20(9.30)	6(6.82)	0.485
25-36	11(6.55)	5(4.67)	6(9.84)	0.203
37-48	3(2.50)	3(4.55)	0(0.00)	<0.001
49-60	1(1.15)	0(0.00)	1(2.33)	<0.001
≥60	2(0.51)	2(1.33)	0(0.00)	<0.001

4.9 Protozoa infection and symptoms

Thirty one percent (31%) of the children who reported with symptoms of diarrhoea, and 26% with symptoms of vomiting were infected with *Giardia lamblia* (**Figure 8**).

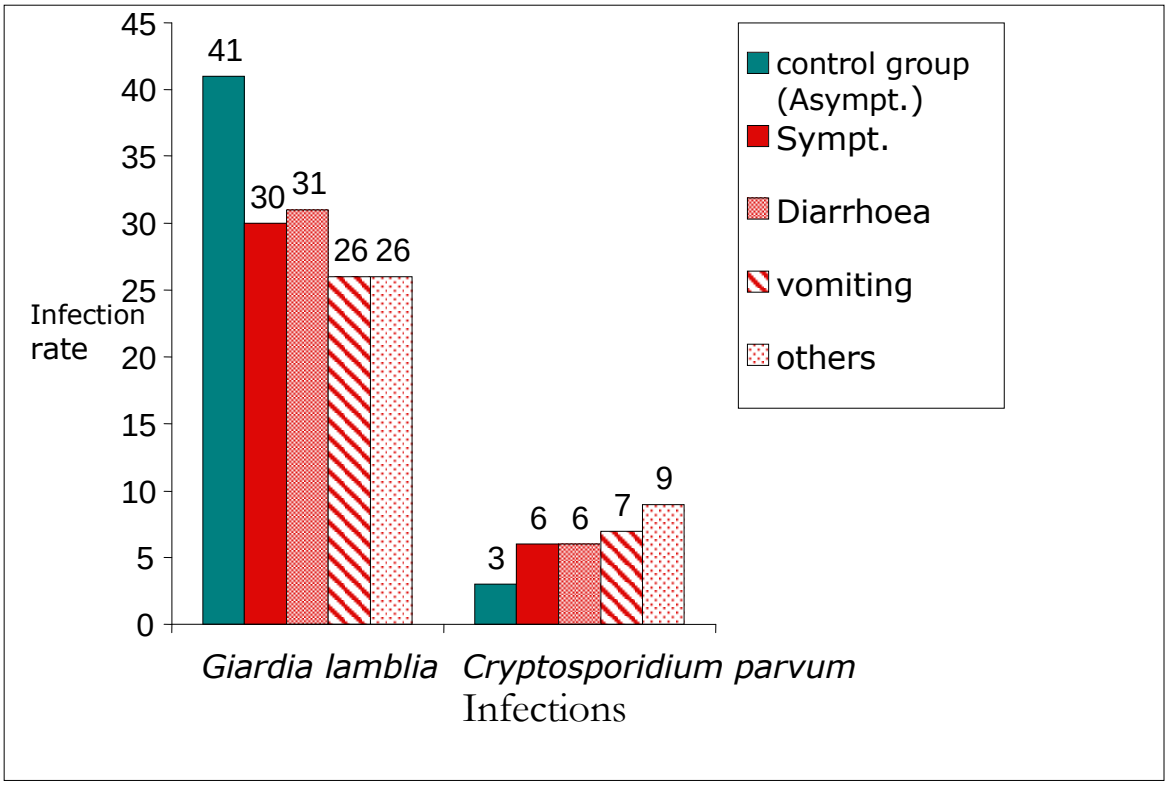


Figure 4.3: Infection rate of protozoa and symptoms by PCR

The infection rate of *Cryptosporidium parvum* in children with vomiting was found to be (7%), lower than those with diarrhoea (9%). There were more *Giardia lamblia* infections in asymptomatic children than with *Cryptosporidium parvum* infections (**Figure 4.3**).

4.10 Stool consistency and *Giardia lamblia* detected by PCR

Giardia lamblia was detected in all the forms (consistencies) of stool samples presented by the symptomatic children. The highest cases of *Giardia lamblia* was detected in firm stools (63.6%) whiles the lowest cases were detected in bloody stools (20%) presented by the symptomatic children. The highest *Giardia lamblia* cases in asymptomatic children were in mucoid stools but there was no case of *Giardia lamblia* in bloody stool. *Giardia lamblia* in the stool samples of asymptomatic

children showed a gradual increase from hard/firm (33.3%), loose (44.4%) watery (54.6%) to mucoid (57.1%) (**Figure 4.4).**

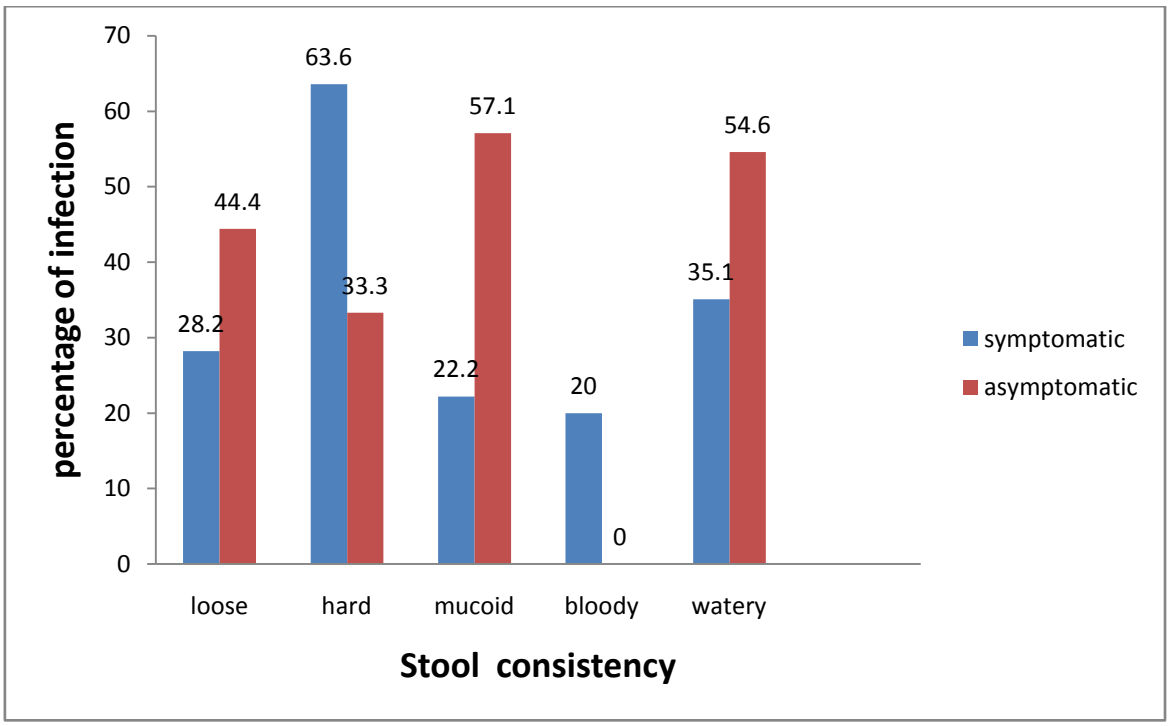


Figure 4.4: Stool consistency and proportions of *Giardia lamblia* parasites detected by PCR

4.11 Stool consistency and *Cryptosporidium parvum* detected by PCR

There was no *Cryptosporidium parvum* detected in the hard/firm and bloody stool samples. However there was fairly uniform distribution of the protozoa in the loose (34%), mucoid (26%), and the watery (21%) samples submitted by the symptomatic children. *Cryptosporidium parvum* could only be detected in loose (0.7%) and watery (9.1%) samples from the asymptomatic children (**Figure 4.5**).

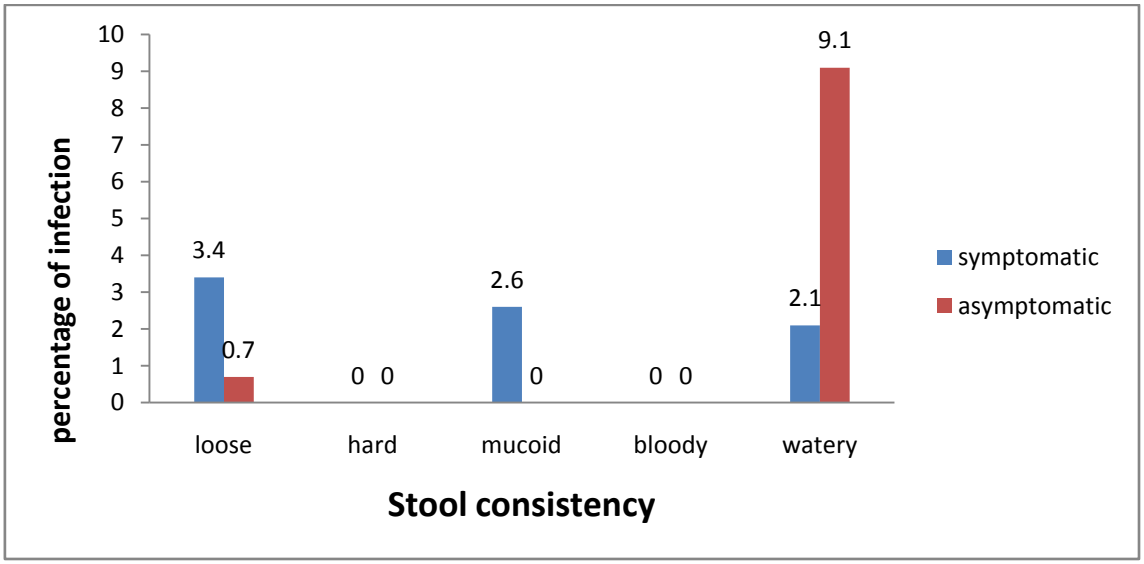


Figure 4.5: Stool consistency and proportion of Cryptosporidium parvum parasites detected by PCR

4.12 Comparision of PCR and Microscopy for the detection of Giardia lamblia

Out of the 1,216 stool samples examined, 413(33.97%) had *Giardia lamblia* using PCR assay whilst 194(15.97%) were positive by microscopy (**Table 4.9**).

Table 4.9: Comparison of PCR and microscopy in the detection of *Giardia lamblia* infection in the study population

	Microscopy		
PCR	**Positive (%)**	**Negative (%)**	**Total**
Positive	180(92.78)	233(22.80)	413(33.96)
Negative	14(7.22)	789(77.20)	803(66.04)
Total	194	1,022	1,216

4.13 Comparison of PCR and Microscopy in the detection of *Cryptosporidium parvum*

Out of a total of 898 stool samples examined for *Cryptosporidium parvum,* 44(4.91%) were positive by microscopy whilst 48(5.35%) were positive by PCR assay (**Table 4.10**).

Table 4.10: Comparison of PCR and microscopy in the detection of *Cryptosporidium parvum* infection in the study population

	Microscopy		
PCR	**Positive (%)**	**Negative (%)**	**Total**
Positive	28(63.64)	20(2.34)	48(5.35)
Negative	16(36.36)	834(97.66)	850(94.65)
Total	44	854	898

4.14 Performance characteristics of Microscopy and PCR in the detection of protozoa with an Expanded Gold Standard

By employing an expanded gold standard (microscopy and PCR positives, PCR only and microscopy only positives), the performance characteristics of microscopy and PCR for *Giardia lamblia* and *Cryptosporidium parvum* are shown in **Table 4.11**. The kappa values were 0.68 (CI=0.64-0.72) and 0.76 (CI=0.69-0.84) respectively.

Table 4.11: Performance characteristics of microscopy and PCR in the detection of protozoa with an expanded gold standard

Expanded gold standard	*Giardia lamblia*		*Cryptosporidium parvum*	
	Microscopy	**PCR**	**Microscopy**	**PCR**
Sensitivity	65.2	96.9	76.2	80.0
Specificity	98.7	81.6	98.2	97.8
+Predictive value	96.8	56.1	91.4	94.12
-Predictive value	81.6	98.6	97.8	98.2

4.15 Comparison of Single and Dual infection of protozoa parasites (*Giardia lamblia* and *Cryptosporidium parvum*) in asymptomatic and symptomatic children

There were 43% symptomatic children with only *Giardia lamblia* infestation whiles 57% were found in the asymptomatic children. *Giardia lamblia* concomitant association with *Cryptosporidium parvum* in symptomatic children was 60% (**Figure 4.6**).

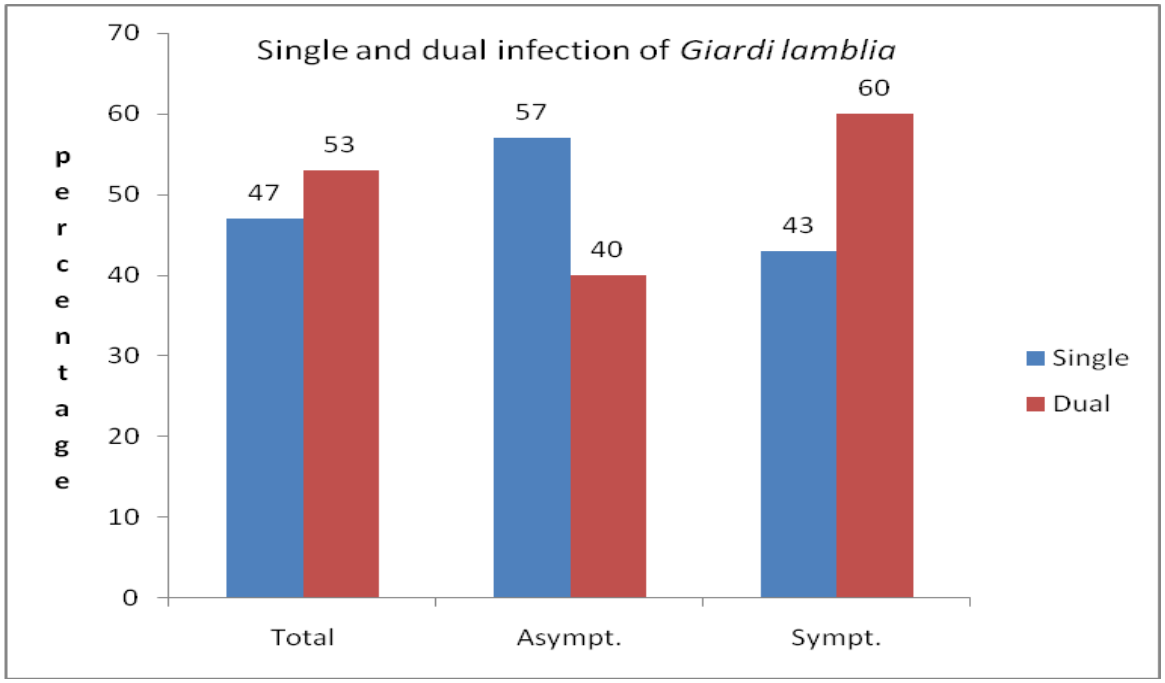


Figure 4.6: Single and dual infections of *Giardia lamblia* in asymptomatic and symptomatic children

Single *Cryptosporidium parvum* infected children with symptoms was 92%. In the asymptomatic children *Cryptosporidium parvum* single infection was 8%. Thirty eight percent (38%) of the asymptomatic children infected with *Cryptosporidium parvum* also had *Giardia lamblia.*

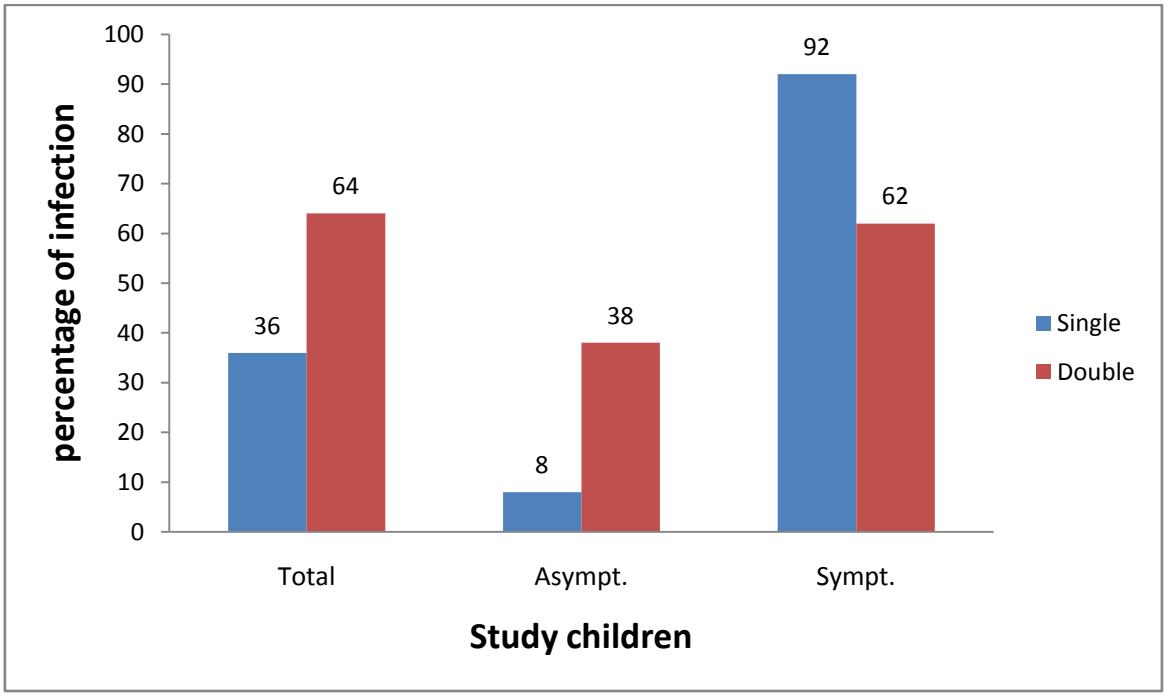


Figure 4.7: Single and dual infections of *Cryptosporidium parvum* in asymptomatic and symptomatic children.

Chapter 5

DISCUSSION

5.1 Discussion

In spite of the advances made in laboratory diagnosis, the prevalence of protozoan infection and their disease burden remain high. *Entamoeba histolytica*, *Giardia lamblia* and *Cryptosporidium parvum* are intestinal and extra intestinal parasites responsible for significant morbidity and mortality, mainly in developing countries.

Accurate identification and differentiation of these protozoa parasites is crucial to clinical management of patients and to epidemiological investigation of outbreaks. Nucleic acid based differentiation has proven to be adequate for this purpose, and the use of real-time PCR has further enhanced it as a diagnostic tool. The present study assessed the use of multiplex real-time PCR assay and microscopy for the detection of the three major enteropathogenic protozoa parasites in children presenting to the Agogo Presbyterian Hospital in the Ashanti Akyem North district of the Ashanti Region of Ghana.

5.2 Protozoa and helminths infections in study children

An important finding of this study was the high prevalence of enteric protozoa (37.9% by PCR, 38.0% by microscopy) and the relatively low rate of helminth infections (2.4%) by microscopy. Also remarkable in the finding was the high prevalence of asymptomatic (2.1%) children with protozoan infection in the district. The low helminth infection rate in children in the district could be associated to the school health program embarked upon by the district health directorate. Children up to 2 years of age to junior high school whilst undergoing the quarterly sight and hearing screening program, are given one (1) tablet of mebendazol (500mg) as a dewormer. This could have contributed to the low rate of helminth infection among the study population.

5.3 *Giardia lamblia* infection in study children

As shown in **Table 4.4** the commonest infestation was giardiasis. The overall prevalence of *Giardia lamblia* in asymptomatic children was 40.3% (**Table 4.5**). Similarly a rate of 31.2% was observed in the symptomatic children. These results are in sharp contrast with the 5% symptomatic and 13% asymptomatic prevalence reported from northern Ghana by Klaus *et al.,* (2007). This low finding from their study could be due to the fact that the stool antigen assays (MeriFluor Crypto & Giardia, Meridian Bioscience, USA) and the PCR employed in their study had low sensitivities compared to the Multiplex Real Time PCR (RT-PCR). The RT-PCR method used in the present study has been shown to be more sensitive and specific (Verweij *et al.*, 2004) than the stool antigen assay and the traditional PCR used in the previous study. Also the previous study was conducted during the dry season (November to January) when protozoa and helminths were comparatively rare. Studies have shown that cysts of *Giardia* known to be waterborne protozoa (Marshall *et al.*, 1997) are washed into water bodies during the raining season thus infection occurrence is high during the rainy season (Vargas *et al.*, 2004). The previous study lacked data during the rainy season. Nevertheless the prevalence of *Giardia lamblia* in the present study falls within the 20 - 40% prevalence range for developing countries reported by Vandenberg (2006).

In consonance with other studies (Meyer, 1990; Hoge *et al.*, 1995; Newman *et al.*, 2001; Klaus *et al.*, 2007), this study showed a high prevalence of *Giardia lamblia* in asymptomatic children. This suggests that *Giardia lamblia* infection either presents sub-clinically or the protozoa have limited pathogenicity. It could also imply that the majority of the children in the district are asymptomatic carriers of a non-pathogenic strain. Even though they look identical when viewed through the microscope, there are different genotypes of *Giardia*. These genotypes are referred to as Assemblages. In a case control study conducted in Bangladesh, Haque *et al.,* (2005) observed that zoonotic genotypes of *G. lamblia* infection (predominantly Assemblage A) was associated with diarrhoea whilst the non zoonotic genotypes

*(*Assemblage B) infection was associated with asymptomatic infection. They found that *Giardia lamblia* assemblage B occurred at a significantly higher rate compared to the assemblage A. This study did not speciate between the *Giardia lamblia* assemblages.

Studies have shown that transmission of *Giardia lamblia* from the environment to humans and from person to person is favoured by high temperatures and moist climatic conditions (Naumova *et al*., 2007), poor personal hygiene and unsanitary habits of individuals (Trauba *et al*., 2009). Domestic animals such as dogs which are reservoir hosts for *Giardia lamblia* (Predominantly Assemblage A) provide the greatest risk of zoonotic transmission of the diplomad *(*Traub *et al.,* 2004*;* Lalle *et al*., 2005*;* Trauba *et al.,* 2009*).* These conditions favourable for transmission of both Assemblages A and B are not uncommon in the study area. The Ashanti Akyem North District is located within the rain forest belt of the Ashanti region. The major occupation of the people is subsistence farming and animal husbandry. Majority of households have domestic animals such as dogs and sheep which are allowed to roam outdoors unsupervised and often observed to mingle with animals from other households. Due to lack of portable water on their farms, the farmers may drink from contaminated streams and rivers. These might have accounted for the high prevalence rate of *Giardia lamblia* infection observed in the symptomatic and the asymptomatic children in the study population.

In the present study, prevalence of *Giardia lamblia* infection was relatively low before aged 12 month but increased thereafter with age. The infection was significantly common in children age over 12 months. It peaked between 12-36 months and then declined thereafter. This finding supports the suggestion that Giardasis is highest among children at the pre-school age who are usually in child care settings. These children acquire immunity after the initial infections in early life which results in some protection in later life (Heresi and Cleary, 1997).

5.4 *Cryptosporidium parvum* infection in study children

Although Cryptosporidiosis is becoming most prevalent among immunodeficient individuals in both developed and developing countries (Haque, 2007), it is not uncommon to find *Cryptosporidium* infection in infants/children with a normal immune status in whom the infections may be either asymptomatic or self limited (Sanchez-Vega *et al*., 2006). In this study 5.6% and 2.4% (by PCR) of symptomatic and asymptomatic respectively recruited children were found to be infected with *Cryptosporidium parvum*. The overall prevalence of 4.4% and 4.9% by PCR and microscopy respectively fall within the lower range of the 5-10% worldwide prevalence put forward by Vandenberg, (2006). Data from 16 case-control studies from 1994 to 2003 indicate that the average overall prevalence of infection in immunocompetent patients in developing countries was 12.7% in those with diarrhea and 4.5% carry the parasite asymptomatically (Bushen *et al*., 2006). In a cross sectional survey of children with diarrhoea conducted in Liberia, Hojlyng *et al.,* (1984) obtained a *Cryptosporidium parvum* prevalence of 26.9%. Addy and Aikin-Bekoe, (1986) in Kumasi Ghana obtained a 12.9% cryptosporidiosis prevalence in symptomatic children aged 2 to 5years. In another study conducted at the Korle-Bu Teaching Hospital in Accra, rates of 27.8% and 15.6% Cryptosporidiosis for symptomatic and asymptomatic children respectively were observed (Adjei *et al*., 2004). The 5.6% for symptomatic and 2.4% for asymptomatic *Cryptosporidium parvum* prevalence obtained in the current study with the real time PCR which is more sensitive suggests a comparatively low rate. This could imply that the protozoon is not endemic in the study children. Although *Cryptosporidium hominis* (genotype I) and *Cryptosporidium parvum* (genotype II) are the two main species of *Cryptosporidium* that infect humans (Haque, 2007), up to nine molecularly distinct types of *Cryptosporidium* have been found to infect HIV-positive individuals as well as immunocompetent children and adults (Ramirez *et al*., 2004). The primers used in the current study were aimed at detecting *Cryptosporidium parvum* (type II). This is because *C. parvum* is known to infect almost all mammals, including humans,

and is a major pathogen of calves. Humans are infected with *C. parvum* in a zoonotic cycle (Fontaine and Guillot, 2003; Hashim *et al.*, 2006). This has resulted in most work on the biology and pathogenicity of *Cryptosporidium* in relation to human infection been done with *C. parvum (*Hashim *et al.,* 2006*)*. Compared to *C. parvum* little is known of the biology of invasion of this human-restricted *C. hominis (*Morgan-Ryan *et al.,* 2002*).* The low prevalence of *Cryptosporidium parvum* observed in the current study could be attributed to the non homogeneous distribution of the *Cryptosporidium* oocysts in stool specimens which could affect the detection rate of the protozoon. It could also possibly mean that *Cryptosporidium parvum* is not a major diarrhoeal causing infectious agent among the study children.

This study has demonstrated that *C. parvum* infection is predominantly common among children less than 5 years and is detected more frequently from symptomatic children than asymptomatic indicating that children with diarrhoea and/or vomiting are more likely to be infected with the protozoa. Cryptosporidiosis was significantly common among children less than 12 months ($p=<0.001$). This is consistent with a previous study in Kumasi, Ghana by Addy and Aikins Bekoe *et al.*,(1986) and in Egypt by Abdel-Messih *et al.,* (2005). However these findings contradict the findings of Gatei *et al.,* (2006) in Kenya and Adjei *et al.,* (2004) at the Korle-Bu Teaching Hospital in Accra who indicated that *Cryptosporidium* infections were highest among children 1–2 years of age. In all previous studies discussed, and this present study the prevalence rates then decreased after two years. The differences in susceptibility by age of children have been attributed to the prevailing subtype of *Cryptosporidium* species endemic in the specific geographical area (Abdel-Messih *et al.*, 2005). All these studies corroborates with results from studies conducted by Chacín-Bonilla *et al.,* (2008) in Venezuella which suggested that contact with contaminated soil in the backyards or around homes without sanitary facilities by children, extreme poverty and

crowding which facilitate fecal-oral transmission were important sources of infection of *Cryptosporidium parvum* in developing countries.

5.5 *Entamoeba histolytica* infection in study children

Although *Entamoeba histolytica* has been reported to be endemic in developing communities with rates up to 10% (Marshall *et al*., 1997), the present study has shown that *Entamoeba histolytica* has low prevalence in the study children. In the present study a total of 9(0.74%) *Entamoeba histolytica/dispar* prevalence was observed by microscopy but *Entamoeba histolytica* and *Entamoeba dispar*-specific DNA amplification using real-time polymerase chain reaction identified only one(1) *E. histolytica* case and revealed a considerably higher prevalence of *Entamoeba dispar* (6.4%). This observation compares well with results obtained in a similar study conducted in northern Ghana by Verweij *et al.,* (2003). The results from the previous study showed a high prevalence (39.8%) of *E. histolytica/dispar* complex by microscopy and 82.8% of *Entamoeba dispar* but only one case of *Entamoeba histolytica* by PCR. These results agree with Zaki and Clark, (2001) that even in areas where invasive amebiasis is suggested to be common, *E. dispar* is by far the more prevalent species. Further investigations are needed to determine other potential *Entamoeba* pathogens in the study children. Studies in Bangladesh (Ali *et al*., 2003), India (Parija and Khairnar, 2005) and Australia (Fotedar *et al*., 2007) have highlighted the prevalence of *E. moshkovskii* infection among children with dysentery and other symptoms.

5.6 Symptomatic and asymptomatic infections in study children

The current study showed high rates of symptomatic and asymptomatic protozoa infection among the study children. It is known that the degree of pathogenicity is related to the distinct immunologic, serologic and genetic identity of the demes constituting the species of parasites involve. It could also be due to long-term suppressive therapy (Current and Garcia, 1991). Studies have shown that long term asymptomatic carriage may represent a form of tolerance to the parasite in

children building up their immune response (Kun *et al.*, 2002). In this way, asymptomatic carriage would protect these children from developing attacks, by keeping their immunity effective (Farnert *et al.*, 1999; Franks *et al.*, 2001). Conversely, asymptomatic carriage may represent a mode of entry to symptomatic infection especially in young children (Henning *et al.*, 2004). Although the study environment (contaminated soil in the backyards or around homes without sanitary facilities, extreme poverty, crowding which facilitate fecal-oral transmission, poor personal hygiene and unsanitary habits of individuals, presence of domestic animals, high temperature and moist) presents favourable conditions for the protozoa infection, further study will be required to ascertained the protective effect or otherwise of the asymptomatic infection among these study children.

5.7 Co-morbidity of infections of *Giardia lamblia* and *Cryptosporidium parvum* in the study children

Multiple parasite infections are prevalent across diverse communities in developing countries (Brooker *et al.*, 2000; Tchuem Tchuente *et al.*, 2003; Raso *et al.*, 2004). The association between the presence of *Giardia lamblia* and *Cryptosporidium parvum* in the intestinal tract of immunocompetent and immunosupressed individuals have been reported (Skeels *et al.*, 1986; Raso *et al.*, 2004). Results from the current study showed that 60% of the symptomatic children with *Giardia lamblia* infection also had *Cryptosporidium parvum*. Whilst Thirty eight percent (38%) of the asymptomatic children infected with *Cryptosporidium parvum* showed *Giardia lamblia* concomitant infection. The reason for this association is unclear, however Wolfson *et al.*, (1984) suggested that this association might represent simultaneous infection from the same source, or predisposition of the host by infection with one parasite toward superinfection or colonization by the other. The exposure levels of these protozoa are related to the environmental and socio-economic characteristics of the study population. Factors affecting zoonotic and anthropometry transmissions of *Giardia lamblia* and *Cryptosporidium parvum* include, an opportunity for animal contact, levels of enzooticity in local animal

populations, poverty, overcrowding, sanitary practices and the degree of person-to-person transmission (Skeels *et al.*, 1986). Thes factors reflect the rates of co-morbidity of infection among these study children.

5.8 Performance characteristics of PCR and Microscopy compared with an Expanded Gold Standard

Compared to PCR, previous studies have found low to moderate sensitivities of conventional microscopy in the detection of the protozoan parasites. Morgan *et al.,* (1998) obtained 83.7% sensitivity and 98.9% specificity when they compared the performance of microscopy to PCR on 115 stool samples for the detection of *Cryptosporidium*. Sodeman and William (1990) reported that direct examination of stools with or without iodine stain establishes the diagnosis of *Giardia lamblia* in up to 70-85% of cases after two stool examinations. He also found the sensitivity of the acid-fast stain for oocysts of *Cryptosporidium* approximately to be 30%. Schuurman *et al.,* (2007) obtained 99% sensitivity and 97% specificity when they compared the performance of microscopy to PCR and a rapid immunoassay for the detection of *Giardia lamblia*. Janoff *et al*., (1989) reported 46-95% sensitivities for *Giardia lamblia*.

Even though nucleic acid methods have been proven to have high analytical sensitivity (the smallest amount of a substance that can be accurately measured in a biological sample) and analytical specificity (the assay's ability to measure a particular organism or substance, rather than another, in a sample) (Saah and Hoover, 1997), they are not without limitations (Malhotra-Kumar *et al*., 2005). They are faced with the risk of cross-contaminations and nonspecific DNA fragments from the environment and clinical samples creating a significant problem that often results in false-positive results (Wang *et al*., 2004; Malhotra-Kumar *et al*., 2005). Damage to cysts/oocyst prior to extraction which may contain little or no intact target DNA, the failure to amplify a single diagnostic sequence due to inhibitors in the sample and possible mutations in the primer binding region may result in false-negative results (Wang *et al*., 2004; Malhotra-Kumar *et al*., 2005).

These limitations invariably affect the diagnostic sensitivity (the percentage of persons who have the disorder of interest who have positive results on the assay) and specificity (the percentage of persons who do not have the condition of interest who have negative results on the assay) of the molecular methods (Saah and Hoover, 1997).

A second gold standard was employed to incorporate more true positives in the study. This was defined as microscopy and PCR positives, PCR only and microscopy only positives. By using this expanded gold standard the sensitivity and specificity of PCR for the detection of *Giardia lamblia* was observed to be 96.9% and 81.6% respectively. It is expected that a highly analytical sensitive assay like the multiplex PCR employed in the current study should more readily translate into high diagnostic sensitivity but this is not always so (Saah and Hoover, 1997). This apparent contradiction results from the shortcomings of sample volume, variations in the clinical spectrum of disease and possible difficulties with specimen preparation and technical performance of the assay leading to the limitations of the assay listed above (Saah and Hoover, 1997). The high false-positive reactions observed (22.8%) reduced the diagnostic specificity. The results therefore agrees with Saah and Hoover (1997) who suggested that assays with high analytical sensitivity and specificity will almost certainly not perform at these very high levels diagnostically. The sensitivity and specificity of microscopy for the detection of *Giardia lamblia* was observed to be 65.2% and 98.7% (kappa = 0.68, CI=0.64-0.72). This shows a fair agreement between microscopy and PCR in the detection of *Giardia*. Sensitivities of 76.2% and 80% and specificities of 98.2% and 97.2% for microscopy and PCR respectively for the detection of *Cryptosporidium parvum* were also observed. There was a very good agreement between the two techniques (kappa = 0.76, CI=0.69-0.84).

Whilst the findings of the current study may differ from results of some previous studies, the present study results concur with other reports (Blessmann *et al.*, 2002;

Morgan-Ryan *et al.*, 2002; Verweij *et al.*, 2004; Haque, 2007) which suggest that Multiplex real time PCR assay for the detection of intestinal protozoa parasites is more sensitive, cable of differentiating closely related species and heterogeneity within species, easier to interpret and very adaptable to batch analysis.

Chapter 6

CONCLUSIONS

6.1 Conclusions

This present study showed high rates of protozoa and relatively low rates of helminth infections in the study population. This study showed that *Giardia lamblia* was the most frequently detected pathogen. However the prevalence of *Giardia lamblia* and the other parasitic pathogens detected amongst the symptomatic and asymptomatic children were found to be similar; thus the significance of these pathogens as diarrhoeal causing agents in the study population is unclear.

This present study has also demonstrated that the multiplex real time PCR assay performs better compared to microscopy in the diagnosis of the intestinal protozoa parasites. However a definite determination of the performance and the validity of both the PCR and microscopy will be dependent on an external quality control employing samples of known content.

6.2 Limitations

There are inherent limitations associated with the study. The sensitivity of microscopic diagnosis of intestinal protozoan disease to a large extent depends on the technique used in the morphological identification of the trophozoites as well as the cysts of the parasites within stool samples. It is also dependent on the number of stool samples examined, the consistency of parasite shedding and the quantity of parasites excreted per sample. Even though the SAF solution preservative and the Formol-ether concentration technique were employed in this study, results obtained were based on a single specimen examination from the study children. Studies have shown that the sensitivity may be increased by analyzing multiple stool samples. The application of the Triple-Faeces-Test (TFT) in a study by Vandenberg *et al.,* (2006) saw a significantly increased recovery of intestinal parasitic infections from 10.3% to 28.15%.

One major setback of the SAF preserved concentration processes is the distortion and destruction of the free form of the amoebae and exudates (Washington Winn *et al.*, 2006). Therefore, estimations of this study could fall short of stool samples containing only these protozoa.

The present study employed the use of diethyl ether as faecal fat solvent in the formol-ether concentration technique. However evaluation of diethyl ether and ethyl acetate (Bukhari and Smith, 1995) has shown that, the former appeared to be more effective at extracting fats from stool samples and yielded cleaner fecal pellets. This was found especially important for detecting *Cryptosporidium* oocysts, which because of their small size (4 to 6 mm), can be easily occluded by contaminating fecal debris.

Another important limitation of the concentration technique is the possible loss of parasites. Weber *et al.,* (1991) detected oocysts in the gauze used for stool filtration and in the supernatants obtained from the first and second centrifugation steps in a study they conducted. This would lead to under estimation of oocyst, especially in stool samples containing a low oocyst concentration.

The inconsistencies in the rate of parasite shedding from patients and in instances where firm stool samples were produced, there might be very few and or unevenly distributed parasites. Reliable results would require dedication and keenness from the microscopist. These challenges might differ from place to place. Therefore results and for that matter the sensitivity of microscopic examination of stool samples can vary between studies according to the techniques used for diagnosis, skills, patience as well as experience of the microscopist.

6.3 Recommendations

The multiplex real-time PCR has demonstrated that it is a better diagnostic alternative in the detection of protozoa infections in patients. However, a PCR technique is limited in its applications in routine stool examination and should not be a substitute for the direct microscopy stool examination, which screens virtually for all intestinal parasites.

It is recommended that Kinyoun staining technique should be employed in the detection of *Cryptosporidium parvum* as part of routine stool analysis in diagnostic laboratories. Data from these laboratories will provide baseline on *Cryptosporidium parvum* infection rates for future investigations.

The high prevalence of protozoa infection in the district suggests that the children are probably exposed to contaminated water and food products. It is thus recommended that parents and school authorities should educate children on personal hygiene, environmental sanitation and the route of infections of these parasites and their impact on health.

The deworming of school children 2 years and above as part of the school health program might have led to the low rates of helminths infections observed in this study. It is recommended that stakeholders of schools and district health management could include the treatment of intestinal protozoa as part of this health program in the district.

6.4 Future study

Laboratory diagnostic techniques employed in this study were PCR assay and microscopy. However, serologic tests specific for *Giardia lamblia*, *Cryptosporidium parvum* and *Entamoeba histolytica* should be employed together with these other methods for future investigations. This serologic technique is helpful in determining previous exposure to parasite contaminated food or water, since most people with these parasitic infection develop serum antibodies to the parasite.

Studies have shown that immunocompromised humans are susceptible to a wide range of *Cryptosporidium* species infections, however immunocompetent individuals are known only to be infected with the five known human species (*C. parvum* human and bovine genotypes, *C. meleagridis*, *C. felis* and *C. canis* (Guyot et., al 2001; Josef et., al 2002). In future studies a wide range of primers for the PCR assay should be employed in order to determine all the five known human and other *Cryptosporidium* species. This will be an important method for assessing public health importance of *Cryptosporidium* infections and its origin whether animal or environmental and for determining infection and contamination sources.

Further studies could be conducted to determine the differences in the prevalence of *Giardia* assemblages A and B which could provide important epidemiological, surveillance and host data for the management of *Giardia* infections in children.

Further studies should be conducted with samples from the environment and domestic animals to examine for the presence of *Giardia* cysts and *Cryptosporidium* oocysts to evaluate the circulation of these parasites. This could be helpful for preventing secondary infections and studying transmission routes and identifying reservoir hosts.

APPENDIX

6.5 Appendix 1 – Consent Form

STUDY ON THE SIGNIFICANCE OF INTESTINAL PROTOZOAN PARASITES AS DIARRHOEA-CAUSING INFECTIOUS AGENTS IN CHILDREN

CHILD WELFARE CLINIC
Version 5.0 (11/11/2007)

Barcode

OPD number | Inpatient-number | Date ___/___/___

Study child data *Registration*

Is the guardian the child's mother? ○ No ○ Yes ○ Alone

Address/Telephone

Family name _____

Given name _____

Sex ○ Male ○ Female

Date of birth ___/___/___ or age_____ ○ Months ○ Years

Village _____

Mother's ethnicity _____

--> if parents ethnicities differ from each other:

Parent's ethnicity differs from each other? ○ No ○ Yes

Father's ethnicity _____

--> if the guardian is the father:

Mother's name _____ Father's name _____

Mother's age _____ Father's age _____

Mother's occupation _____ Father's occupation _____

Informed consent

We want to collect all the information about the ill children who come to the under fiver clinic and put it on a computer. We will then be able to see what diseases are important in and around Agogo. All the computer information will be anonymous and confidential.

However if we find your child has a certain infection we will inform the doctors in the clinic so you can be prescribed the correct treatment. Sometimes this will be immediately and sometimes this will be some days later.

Do you agree to including your child's information on our computer?
☐ Yes ☐ No

Do you agree to your child providing a stool sample to be tested for different infections?
☐ Yes ☐ No

Do you agree to your child providing a sputum sample to be tested for different infections?
☐ Yes ☐ No

--> If your child is admitted to the paediatric ward:
Do you agree to your child providing a blood sample to look for bacteria in the blood?
☐ Yes ☐ No

Signed/Thumb Print ☐ MOTHER ☐ FATHER ☐ GUARDIAN:

_____ _____ ___/___/___
Sign Guardian Date

Witnessed by

_____ _____ ___/___/___
Sign Name in block letters Date

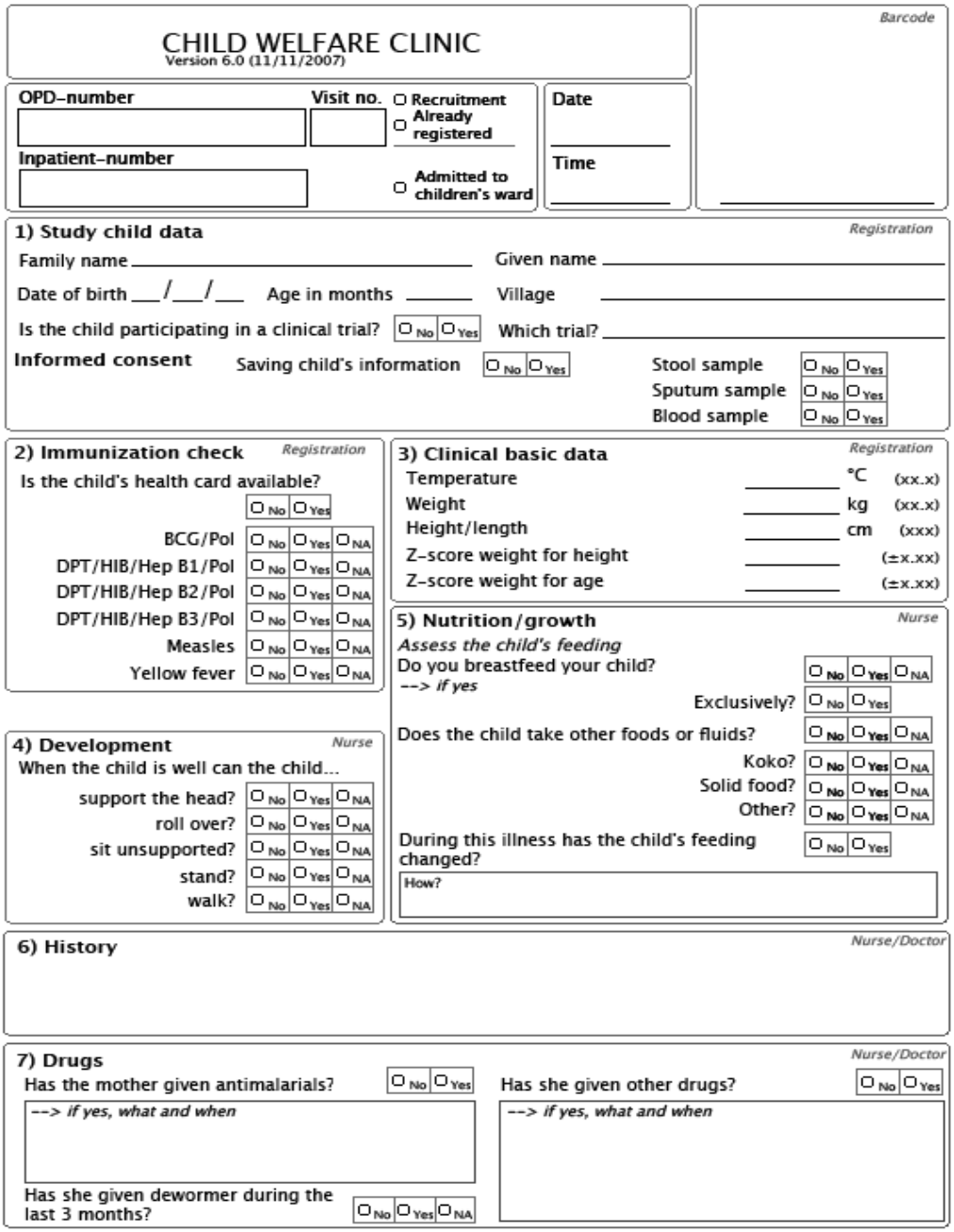
Consent obtained by

_____ _____ ___/___/___
Sign Name in block letters Date

Registering person: _____ Sign:_____

6.6 Appendix 2 – Case Report Form 1

STUDY ON THE SIGNIFICANCE OF INTESTINAL PROTOZOAN PARASITES AS DIARRHOEA-CAUSING INFECTIOUS AGENTS IN CHILDREN



CHILD WELFARE CLINIC
Version 6.0 (11/11/2007)

Barcode

OPD–number | Visit no. | ○ Recruitment ○ Already registered | Date
Inpatient–number | ○ Admitted to children's ward | Time

1) Study child data — Registration

Family name _____ Given name _____
Date of birth ___/___/___ Age in months _____ Village _____
Is the child participating in a clinical trial? ○ No ○ Yes Which trial? _____
Informed consent Saving child's information ○ No ○ Yes
Stool sample ○ No ○ Yes
Sputum sample ○ No ○ Yes
Blood sample ○ No ○ Yes

2) Immunization check — Registration

Is the child's health card available? ○ No ○ Yes

BCG/Pol	○ No	○ Yes	○ NA
DPT/HIB/Hep B1/Pol	○ No	○ Yes	○ NA
DPT/HIB/Hep B2/Pol	○ No	○ Yes	○ NA
DPT/HIB/Hep B3/Pol	○ No	○ Yes	○ NA
Measles	○ No	○ Yes	○ NA
Yellow fever	○ No	○ Yes	○ NA

3) Clinical basic data — Registration

Temperature	°C	(xx.x)
Weight	kg	(xx.x)
Height/length	cm	(xxx)
Z-score weight for height		(±x.xx)
Z-score weight for age		(±x.xx)

4) Development — Nurse

When the child is well can the child...

support the head?	○ No	○ Yes	○ NA
roll over?	○ No	○ Yes	○ NA
sit unsupported?	○ No	○ Yes	○ NA
stand?	○ No	○ Yes	○ NA
walk?	○ No	○ Yes	○ NA

5) Nutrition/growth — Nurse

Assess the child's feeding
Do you breastfeed your child? ○ No ○ Yes ○ NA
--> *if yes*
Exclusively? ○ No ○ Yes
Does the child take other foods or fluids? ○ No ○ Yes ○ NA
Koko? ○ No ○ Yes ○ NA
Solid food? ○ No ○ Yes ○ NA
Other? ○ No ○ Yes ○ NA
During this illness has the child's feeding changed? ○ No ○ Yes
How?

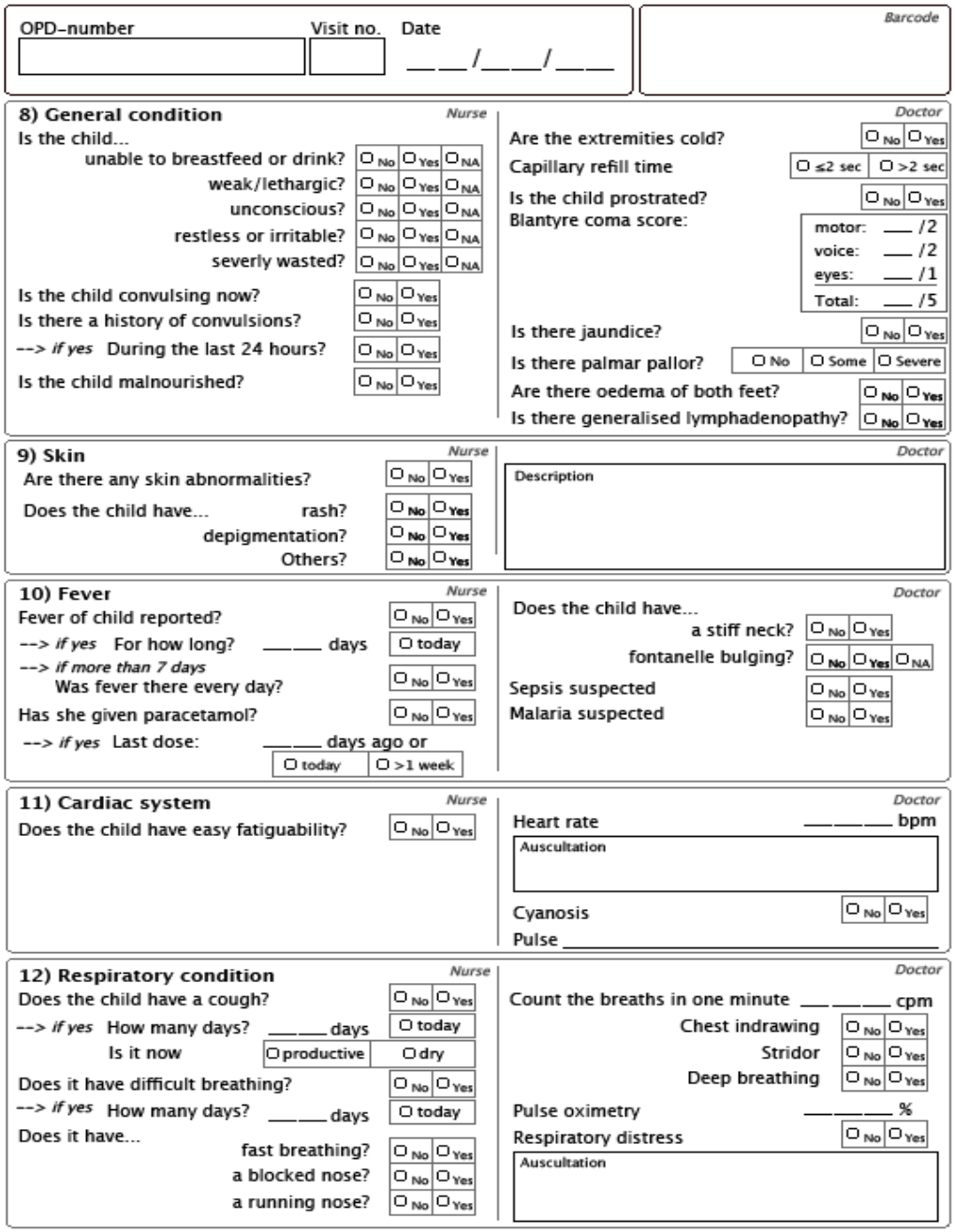
6) History — Nurse/Doctor

7) Drugs — Nurse/Doctor

Has the mother given antimalarials? ○ No ○ Yes
--> *if yes, what and when*
Has she given dewormer during the last 3 months? ○ No ○ Yes ○ NA
Has she given other drugs? ○ No ○ Yes
--> *if yes, what and when*

6.7 Appendix 3 – Case Report Form 2

STUDY ON THE SIGNIFICANCE OF INTESTINAL PROTOZOAN PARASITES AS DIARRHOEA-CAUSING INFECTIOUS AGENTS IN CHILDREN



OPD–number | Visit no. | Date ___/___/___ | Barcode

8) General condition (Nurse)

Is the child...
- unable to breastfeed or drink? No / Yes / NA
- weak/lethargic? No / Yes / NA
- unconscious? No / Yes / NA
- restless or irritable? No / Yes / NA
- severly wasted? No / Yes / NA

Is the child convulsing now? No / Yes

Is there a history of convulsions? No / Yes

--> *if yes* During the last 24 hours? No / Yes

Is the child malnourished? No / Yes

(Doctor)

Are the extremities cold? No / Yes

Capillary refill time ≤2 sec / >2 sec

Is the child prostrated? No / Yes

Blantyre coma score:
- motor: ___ /2
- voice: ___ /2
- eyes: ___ /1
- Total: ___ /5

Is there jaundice? No / Yes

Is there palmar pallor? No / Some / Severe

Are there oedema of both feet? No / Yes

Is there generalised lymphadenopathy? No / Yes

9) Skin (Nurse)

Are there any skin abnormalities? No / Yes

Does the child have... rash? No / Yes

depigmentation? No / Yes

Others? No / Yes

(Doctor)

Description

10) Fever (Nurse)

Fever of child reported? No / Yes

--> *if yes* For how long? ___ days / today

--> *if more than 7 days* Was fever there every day? No / Yes

Has she given paracetamol? No / Yes

--> *if yes* Last dose: ___ days ago or today / >1 week

(Doctor)

Does the child have...
- a stiff neck? No / Yes
- fontanelle bulging? No / Yes / NA

Sepsis suspected No / Yes

Malaria suspected No / Yes

11) Cardiac system (Nurse)

Does the child have easy fatiguability? No / Yes

(Doctor)

Heart rate ___ bpm

Auscultation

Cyanosis No / Yes

Pulse ___

12) Respiratory condition (Nurse)

Does the child have a cough? No / Yes

--> *if yes* How many days? ___ days / today

Is it now productive / dry

Does it have difficult breathing? No / Yes

--> *if yes* How many days? ___ days / today

Does it have...
- fast breathing? No / Yes
- a blocked nose? No / Yes
- a running nose? No / Yes

(Doctor)

Count the breaths in one minute ___ cpm
- Chest indrawing No / Yes
- Stridor No / Yes
- Deep breathing No / Yes

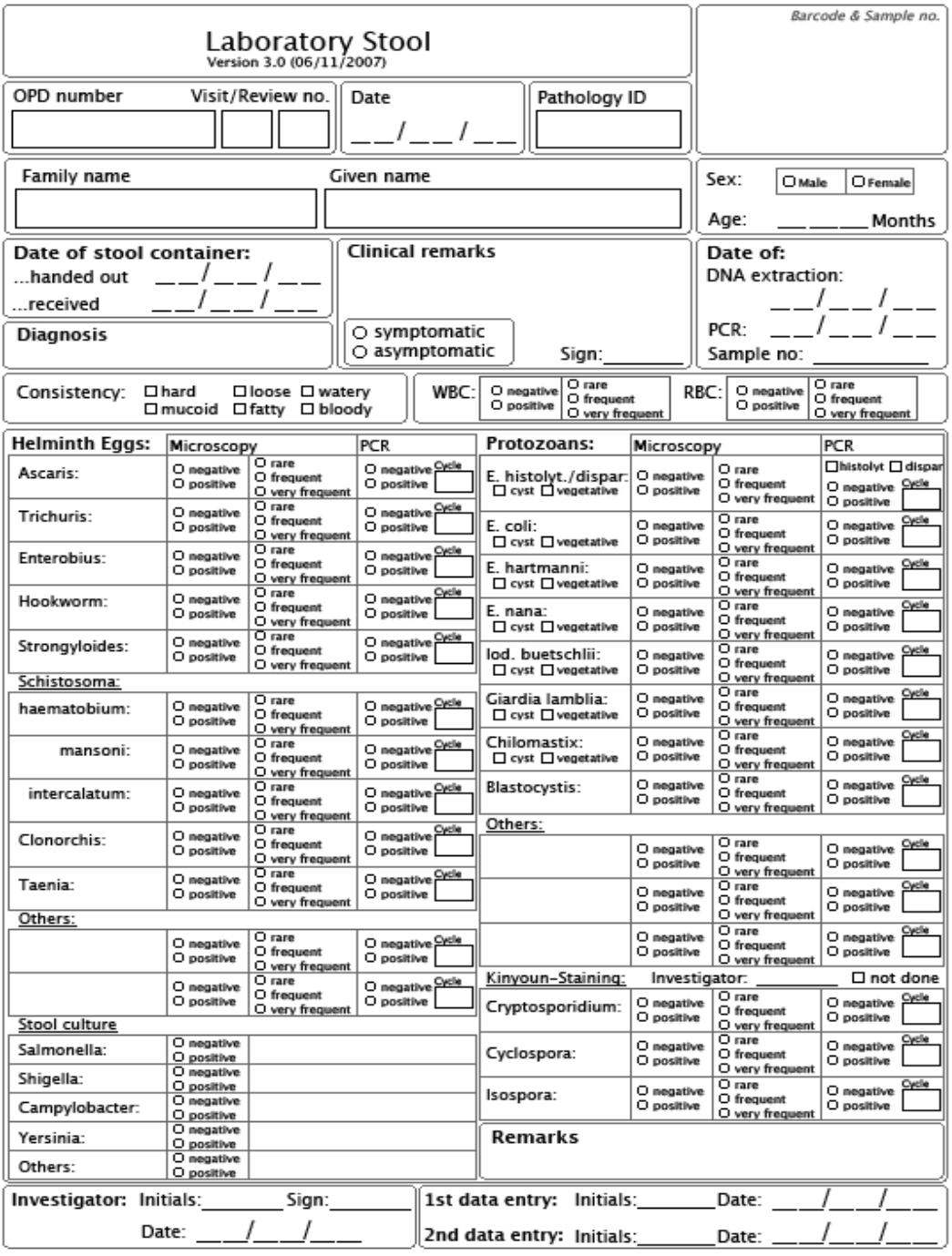
Pulse oximetry ___ %

Respiratory distress No / Yes

Auscultation

6.8 Appendix 4 – Laboratory Result Form

STUDY ON THE SIGNIFICANCE OF INTESTINAL PROTOZOAN PARASITES AS DIARRHOEA-CAUSING INFECTIOUS AGENTS IN CHILDREN



Laboratory Stool
Version 3.0 (06/11/2007)

Barcode & Sample no.

OPD number | Visit/Review no. | Date __/__/__ | Pathology ID

Family name | Given name | Sex: ○ Male ○ Female | Age: ___ Months

Date of stool container:
...handed out __/__/__
...received __/__/__

Clinical remarks
○ symptomatic ○ asymptomatic
Sign:_____

Date of:
DNA extraction: __/__/__
PCR: __/__/__
Sample no: _____

Diagnosis

Consistency: ☐ hard ☐ loose ☐ watery ☐ mucoid ☐ fatty ☐ bloody

WBC: ○ negative ○ positive | ○ rare ○ frequent ○ very frequent
RBC: ○ negative ○ positive | ○ rare ○ frequent ○ very frequent

Helminth Eggs:	Microscopy		PCR
Ascaris:	○ negative ○ positive	○ rare ○ frequent ○ very frequent	○ negative ○ positive Cycle
Trichuris:	○ negative ○ positive	○ rare ○ frequent ○ very frequent	○ negative ○ positive Cycle
Enterobius:	○ negative ○ positive	○ rare ○ frequent ○ very frequent	○ negative ○ positive Cycle
Hookworm:	○ negative ○ positive	○ rare ○ frequent ○ very frequent	○ negative ○ positive Cycle
Strongyloides:	○ negative ○ positive	○ rare ○ frequent ○ very frequent	○ negative ○ positive Cycle
Schistosoma:			
haematobium:	○ negative ○ positive	○ rare ○ frequent ○ very frequent	○ negative ○ positive Cycle
mansoni:	○ negative ○ positive	○ rare ○ frequent ○ very frequent	○ negative ○ positive Cycle
intercalatum:	○ negative ○ positive	○ rare ○ frequent ○ very frequent	○ negative ○ positive Cycle
Clonorchis:	○ negative ○ positive	○ rare ○ frequent ○ very frequent	○ negative ○ positive Cycle
Taenia:	○ negative ○ positive	○ rare ○ frequent ○ very frequent	○ negative ○ positive Cycle
Others:			
	○ negative ○ positive	○ rare ○ frequent ○ very frequent	○ negative ○ positive Cycle
	○ negative ○ positive	○ rare ○ frequent ○ very frequent	○ negative ○ positive Cycle
Stool culture			
Salmonella:	○ negative ○ positive		
Shigella:	○ negative ○ positive		
Campylobacter:	○ negative ○ positive		
Yersinia:	○ negative ○ positive		
Others:	○ negative ○ positive		

Protozoans:	Microscopy		PCR
E. histolyt./dispar: ☐ cyst ☐ vegetative	○ negative ○ positive	○ rare ○ frequent ○ very frequent	☐ histolyt ☐ dispar ○ negative ○ positive Cycle
E. coli: ☐ cyst ☐ vegetative	○ negative ○ positive	○ rare ○ frequent ○ very frequent	○ negative ○ positive Cycle
E. hartmanni: ☐ cyst ☐ vegetative	○ negative ○ positive	○ rare ○ frequent ○ very frequent	○ negative ○ positive Cycle
E. nana: ☐ cyst ☐ vegetative	○ negative ○ positive	○ rare ○ frequent ○ very frequent	○ negative ○ positive Cycle
Iod. buetschlii: ☐ cyst ☐ vegetative	○ negative ○ positive	○ rare ○ frequent ○ very frequent	○ negative ○ positive Cycle
Giardia lamblia: ☐ cyst ☐ vegetative	○ negative ○ positive	○ rare ○ frequent ○ very frequent	○ negative ○ positive Cycle
Chilomastix: ☐ cyst ☐ vegetative	○ negative ○ positive	○ rare ○ frequent ○ very frequent	○ negative ○ positive Cycle
Blastocystis:	○ negative ○ positive	○ rare ○ frequent ○ very frequent	○ negative ○ positive Cycle
Others:			
	○ negative ○ positive	○ rare ○ frequent ○ very frequent	○ negative ○ positive Cycle
	○ negative ○ positive	○ rare ○ frequent ○ very frequent	○ negative ○ positive Cycle
	○ negative ○ positive	○ rare ○ frequent ○ very frequent	○ negative ○ positive Cycle
Kinyoun-Staining:	Investigator: _____		☐ not done
Cryptosporidium:	○ negative ○ positive	○ rare ○ frequent ○ very frequent	○ negative ○ positive Cycle
Cyclospora:	○ negative ○ positive	○ rare ○ frequent ○ very frequent	○ negative ○ positive Cycle
Isospora:	○ negative ○ positive	○ rare ○ frequent ○ very frequent	○ negative ○ positive Cycle

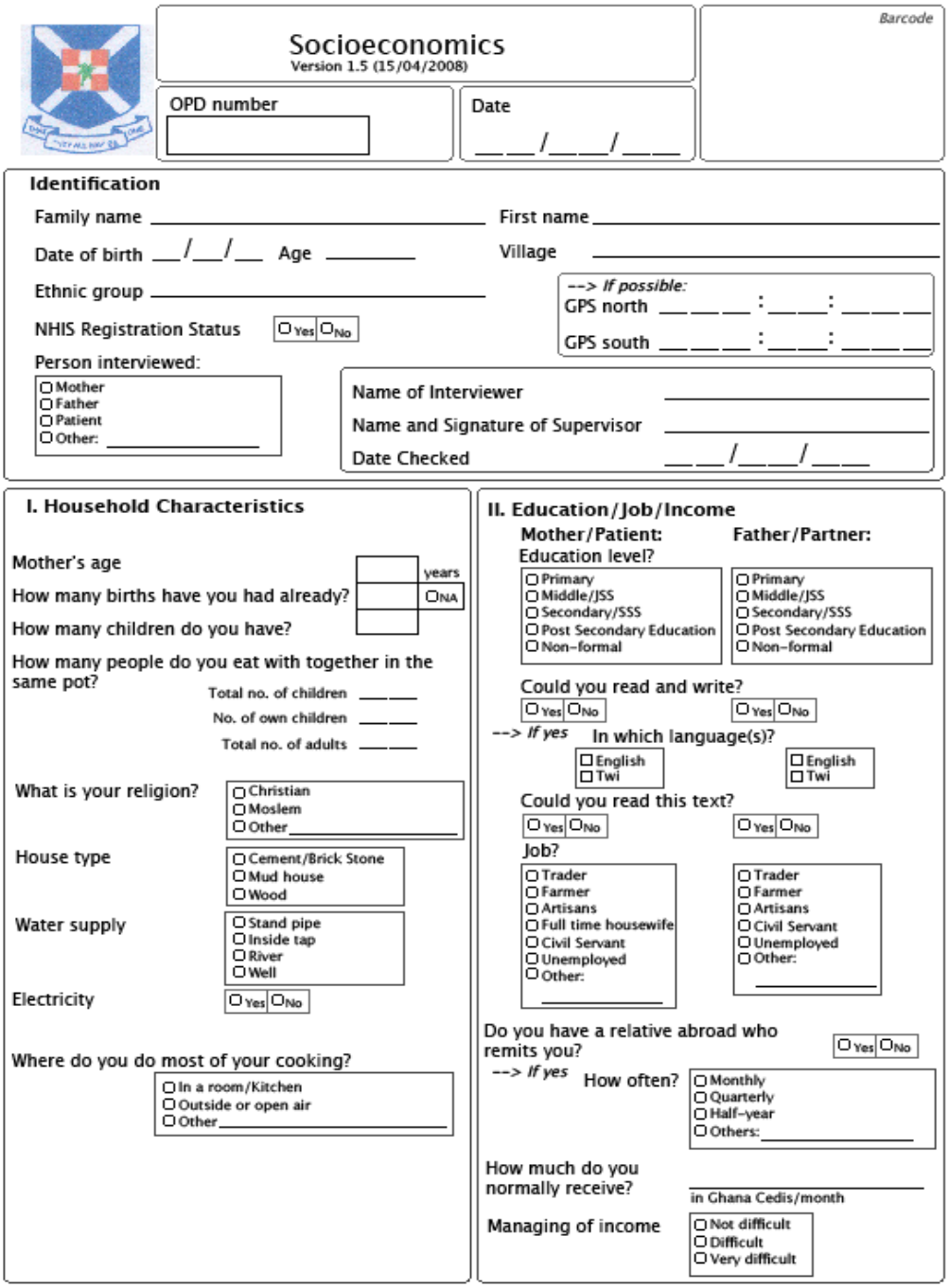
Remarks

Investigator: Initials:_____ Sign:_____
Date: ____/____/____

1st data entry: Initials:_____ Date: ____/____/____
2nd data entry: Initials:_____ Date: ____/____/____

6.9 Appendix 5 – Socioeconomic Report Form

STUDY ON THE SIGNIFICANCE OF INTESTINAL PROTOZOAN PARASITES AS DIARRHOEA-CAUSING INFECTIOUS AGENTS IN CHILDREN



Socioeconomics
Version 1.5 (15/04/2008)

Barcode

OPD number

Date ___/___/___

Identification

Family name _____ First name _____

Date of birth ___/___/___ Age _____ Village _____

Ethnic group _____

--> *If possible:*
GPS north ___ ___ ___ : ___ : ___ ___ ___
GPS south ___ ___ ___ : ___ : ___ ___ ___

NHIS Registration Status ☐ Yes ☐ No

Person interviewed:
☐ Mother
☐ Father
☐ Patient
☐ Other: _____

Name of Interviewer _____
Name and Signature of Supervisor _____
Date Checked ___/___/___

I. Household Characteristics

Mother's age ____ years
How many births have you had already? ____ ☐ NA
How many children do you have? ____

How many people do you eat with together in the same pot?
Total no. of children ___
No. of own children ___
Total no. of adults ___

What is your religion?
☐ Christian
☐ Moslem
☐ Other _____

House type
☐ Cement/Brick Stone
☐ Mud house
☐ Wood

Water supply
☐ Stand pipe
☐ Inside tap
☐ River
☐ Well

Electricity ☐ Yes ☐ No

Where do you do most of your cooking?
☐ In a room/Kitchen
☐ Outside or open air
☐ Other _____

II. Education/Job/Income

	Mother/Patient:	Father/Partner:
Education level?	☐ Primary ☐ Middle/JSS ☐ Secondary/SSS ☐ Post Secondary Education ☐ Non-formal	☐ Primary ☐ Middle/JSS ☐ Secondary/SSS ☐ Post Secondary Education ☐ Non-formal
Could you read and write?	☐ Yes ☐ No	☐ Yes ☐ No
--> *If yes* In which language(s)?	☐ English ☐ Twi	☐ English ☐ Twi
Could you read this text?	☐ Yes ☐ No	☐ Yes ☐ No
Job?	☐ Trader ☐ Farmer ☐ Artisans ☐ Full time housewife ☐ Civil Servant ☐ Unemployed ☐ Other:	☐ Trader ☐ Farmer ☐ Artisans ☐ Civil Servant ☐ Unemployed ☐ Other:

Do you have a relative abroad who remits you? ☐ Yes ☐ No
--> *If yes* How often?
☐ Monthly
☐ Quarterly
☐ Half-year
☐ Others: _____

How much do you normally receive? _____ in Ghana Cedis/month

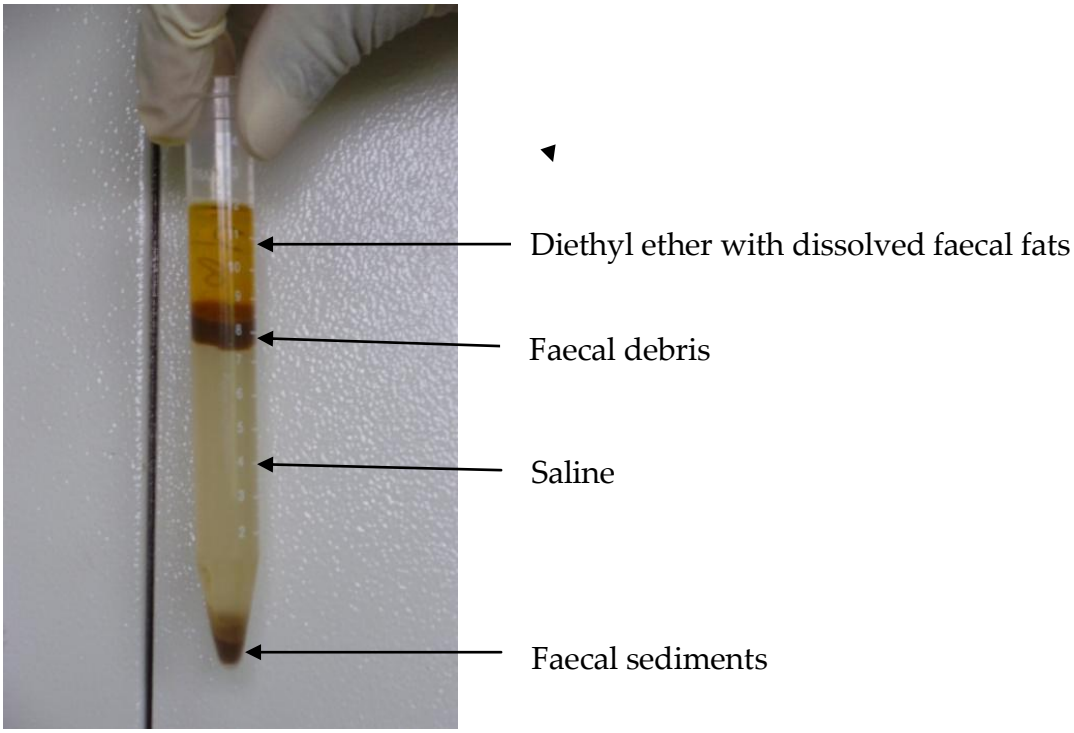
Managing of income
☐ Not difficult
☐ Difficult
☐ Very difficult

6.10 Appendix 6 – Stool Preservation and Preparation

The stool samples were processed as soon as possible after collection from the patient to avoid degeneration of the parasites. The 2ml Eppendof tube was pre weighed on the precision balance. Using the spatula an aliquot of the patient´s stool sample was fill into the pre weighed 2ml tube. The weight was determined and adjusted to 0.2g. The tubes were labelled with the barcode-number. 4ml sodium-acetate formalin (SAF) solution was added to the remaining unpreserved stool sample in the 15ml tube to avoid degeneration of the parasites. The 2ml tubes containing the 0.2g native stool were kept in a labelled box ("Stool PCR") and put into a freezer at –20°C to -80°C. The SAF-preserved stool sample in 15ml tube was Stored at room temperature or continued to the concentration technique.

6.11 Appendix 7 – Formol-ether concentration method

A small sample (the size of a peanut), from the stool sample received was put into a labelled Falcon centrifuge tube and 7 ml SAF-solution added. The tube was thoroughly shaken. Using a funnel the stool sample was poured through gauze into another labelled Falcon centrifuge tube. The preparation was centrifuged at 2000U/min for 1 minute and the supernatant discarded. 7 ml of 0.9% NaCl solution and 2-3 ml Ether was added to the filtrate. The tube was thoroughly shaken for 2-3 minutes keeping the tube close with the thumb. Four (4) layers were obtained after 3 minutes centrifugation at 2000U/min. The top three (3) layers were discarded. The concentrate was divided into two portions. One portion in a 15 ml falcon tube was stained with lugol's iodine. Smear preparation of the other portion on clean dry 76 mm x26 mm microscopy slide for microscopy.



6.12 Appendix 8 – Kinyoun staining method

A thin film from a drop of SAF-stool formol-ether concentrate on a microscope slide was prepared and allowed to air-dry. It was fixed with methanol (100%) for 1 minute. The preparation was subsequently stained for 5 minutes in Kinyoun Carbol Fuchsin staining solution. The stained slide was briefly washed with 50% ethanol and cleaned thoroughly with tap water. Excess stain was removed with 0.5% sulfanic acid for 2-3 minutes. The slide was counterstained with Loffler's methylene blue staining solution for 1 minute. The stained slide was cleaned with tap water and allowed to air dry.

6.13 Appendix 9 – DNA extraction protocol

Equipments used: Microcentifuge, Thermal cycler, Vortexer, Latex Gloves, Pipette (100 µl and 1000 µl) and pipette tips, 1.5 ml and 2.0 ml tubes

Preparations: The thermal cycler was heated up to 70°C. The ASL Buffer and the AL Buffer solutions were put on the thermal cycler to dissolve precipitates completely. The frozen stool samples were taken out of the freezer and ASL Buffer was added immediately before it thawed. 25 ml and 30 ml ethanol was

added to the AW 1 Buffer and AW 2 buffers respectively and mixed thoroughly.

1.4 ml ASL Buffer was added to each stool sample and vortexed for at least 1min until the stool sample was thoroughly homogenized. The suspension was then heated at 70°C for 5min. The samples were vortexed for 15 sec after which there were centrifuged at 13200 U/min for 1min. 1.2ml of the supernatant was pipetted into a new 2.0ml tube and the pellet discarded.

One (1) InhibitEX tablet was then added to each sample and vortexed again immediately for at least 1min (or until the tablet has completely suspended). After 1min incubation at room temperature the samples were centrifuged at 13200 U/min for 3min. The supernatant was pipetted into a new 1.5ml tube and again centrifuged at 13200 U/min for 3min. The pellets were discarded. Fifteen microliter (15µl) Proteinase K, 200µl of the supernatant and 200µl, AL Buffer were added into a new 1.5ml tube and vortex for 15sec. The preparations were then incubated at 70°C for 10min and centrifuged for 10sec. at full speed. 200µl ethanol (96-100%) was added and mixed by vortexing for 10sec. The complete lysate was pipetted into the QIAamp spin column after10sec centrifugation at full speed. The column was centrifuged at 13200U/min for 1min until the complete lysate passes through the membrane and was found in the bottom of the tube. The tube with the filtrate was discarded.

The QIAamp spin column was transferred into a new 2.0ml collection tube. 500µl AW 1 Buffer was added and centrifuged at 13200U/min for 1min. The tube with the filtrate was discarded.

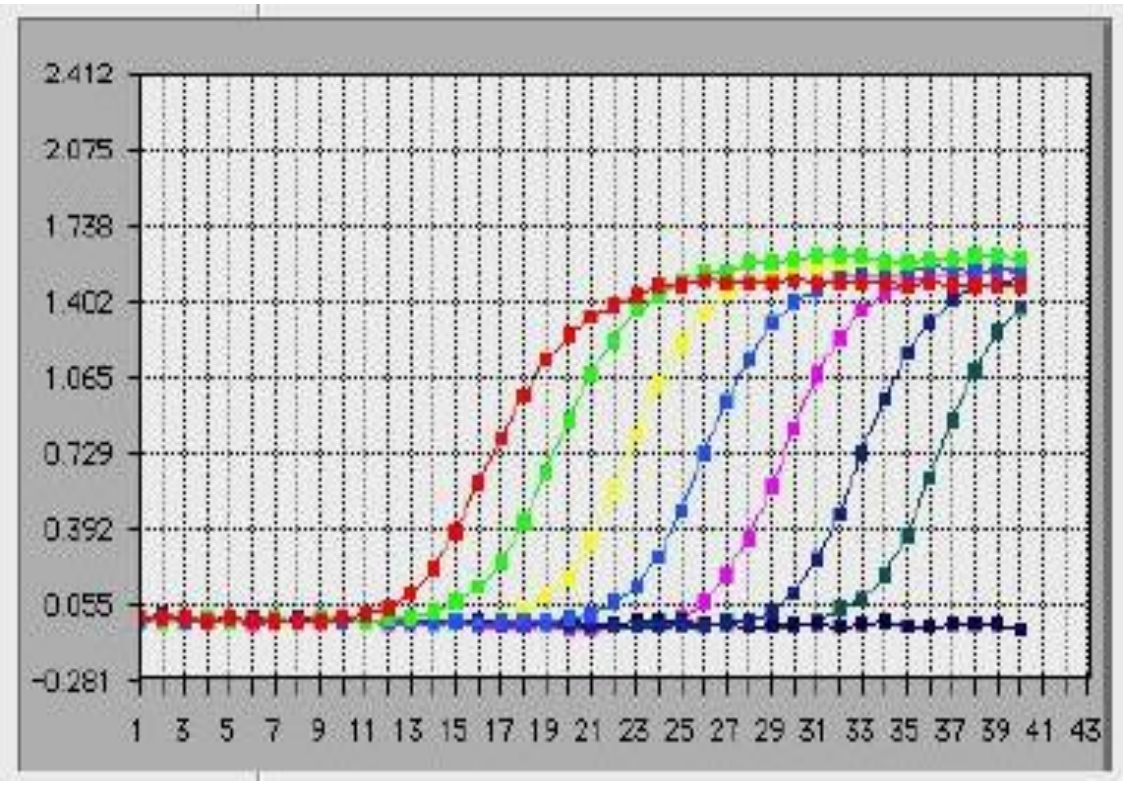
The column was again transferred into a new 2.0ml collection tube. 500µl AW 2 Buffer added and centrifuged at 132000U/min for 3min. The tube with the filtrate was discarded. The spin column was transferred into a 1.5ml tube and the collection tube containing the filtrate discarded.

200µl AE Buffer was pipetted into the spin column incubated for 1min at room temperature and centrifuged at 13200U/min for 1min. The 1.5ml tube containing the final 200µl filtrate (DNA-elution) was labelled and stored at –20°C to -80°C. The QIAamp spin column was discarded.

6.14 Appendix 10 – The Real Time PCR principles

Real-time detection of PCR products is made possible by including in the reaction a fluorescent molecule that reports an increase in the amount of DNA with a proportional increase in fluorescent signal. The fluorescent chemistries employed for this purpose include DNA-binding dyes and fluorescently labeled sequence specific primers or probes. Specialized thermal cyclers equipped with fluorescence detection modules are used to monitor the fluorescence as amplification occurs. The measured fluorescence reflects the amount of amplified product in each cycle (Bio-RadLaboratories, 2006).

REAL-TIME PCR SERIAL DILUTION OF TEMPLATE DNA



Multiplex polymerase chain reaction (PCR) is defined as the simultaneous amplification of multiple regions of DNA templates by adding more than one primer pair to the amplification reaction mixture. Multiplex reactions require the use of multiple reporters to follow each individual amplification reaction. To distinguish each reaction, reporter fluorophores with minimally overlapping emission spectra are chosen (Schoske *et al.*, 2003).

All primers are designed with approximately the same Tm (55–60°C), and all probes with approximately the same Tm (~5–10°C higher than the primers).The different primer and probe sets do not exhibit complementarily to one another because all primers and probes will be present in one reaction (Wittwer *et al.*, 2001). After several (often about 40) rounds of amplification, the PCR product is analyzed.

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