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Epidemiology and Molecular Characterization of *Giardia Lamblia* and *Cryptosporidium* Sp. Infections among Children In Accra, Ghana

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This thesis is submitted to the University of Ghana, Legon in partial fulfillment of the requirement for the award of **PhD Microbiology degree**

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DECLARATION

It is hereby declared that the work in this thesis is original and was carried out by the author. Work from other authors where cited have been duly acknowledged. This work has not been submitted to any institution wholly or partially for the award of any degree.

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DEDICATION

This work is affectionately dedicated to my wife Josephine for her love, support and encouragement. It is also dedicated to Bernard and Michael, our two boys. The Almighty God has brought us a mighty long way. May His name be praised forever, for what He has done.

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ABSTRACT

Giardiasis and cryptosporidiosis remain as part of the commonest gastroenteritis in Ghana. The diseases are caused by the protozoan parasites, *Giardia lamblia* and *Cryptosporidium* sp. respectively. Inadequate supply of treated water and poor sanitation are some of the key factors leading to the spread of these infections. Being zoonotic diseases, it is suspected that, a large proportion of human infections could come from infected domestic and farm animals. Though, use of molecular tools has helped to understand how the diseases spread in humans, animals, and the environment, very little information is available on the epidemiology and transmission routes of *G. lamblia* and *Cryptosporidium* sp. in Ghana, and a genetic characterization of the parasite has also not been thoroughly investigated. Information on clinical manifestations of *Giardia* infections and co-infections with other diarrhoeal causing agents particularly rotavirus remain scanty. In the present study, the epidemiology and molecular characterization of the two parasitic infections were investigated. The study, a prospective cross-sectional hospital and community-based, was conducted in Accra, Ghana. A total of 485 patients comprising of 365 diarrhoeic and 120 non-diarrhoeic children of age ≤ 5 years, were studied. Stool samples were tested microscopically, and by enzyme immunoassay kits. Positive samples were tested by the semi- nested polymerase chain reaction (PCR) and subsequently characterized into genotypes by PCR-RFLP, and nucleotide sequence analysis. Demographic and clinical data were obtained by a structured questionnaire. In the hospital-based study, prevalence rates of 5.8% and 22.0% were observed for *G. lamblia* and *Cryptosporidium* sp. infections respectively, and prevalence in diarrhoeic children was significantly higher than non-diarrhoeic children ($P < 0.0001$). Infection in day care centres was 10.1% for *G. lamblia* and 4.2% for *Cryptosporidium*. Neither gender nor breastfeeding habits, education level of mother, presence of domestic animals, source of children's food, seasons (dry or rainy) was a risk factor for infections of

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the two parasites. However, age and source of drinking water were identified as associated risk factors for infection. *G. lamblia* genotype B and *Cryptosporidium parvum* were identified in the genotyping study. Although severity of rotaviral diarrhoea was reduced by *Giardia* co-infection, the results cannot be conclusive. Although both parasites were present in the studied population, cryptosporidial diarrhoea appears to be more common than giardial diarrhoea. The presence of infections among non-diarrhoeal children is of much concern, as they can spread infections unknowingly. The presence of genotype B as the only prevailing genotype of *G. lamblia* indicates that infections from animals will be uncommon, but *Cryptosporidium parvum* transmission could be either anthroponotic or zoonotic. The co-infection study had a limitation, and therefore demands further investigation. Several *Cryptosporidium* isolates that were successfully sequenced but whose identity were not clear, need further investigation as they could be new species emerging.

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CHAPTER ONE

INTRODUCTION

1.1 General Introduction

Diarrhoeal diseases are common among people in developing countries, and they cause considerable amount of morbidity and mortality, especially among children (Kosek *et al.*,2003; Boschi-Pinto *et al.*,2008). According to the United Nations Children's Fund (UNICEF)/ World Health Organization (WHO) joint report (2009), diarrhoea remains the most common cause of death among children under five globally. The report indicated that each year, about 1.5 million children in this age group die as a result of diarrhoea, and more than 80 per cent of these deaths occur in Africa and South Asia.

Giardiasis and cryptosporidiosis, caused by the protozoan parasites *G. lamblia* and *Cryptosporidium sp.* respectively, are common diarrhoeal diseases in both infants and adults. They are among the most common causes of gastroenteritis in humans worldwide. Symptomatic infection is characterized by diarrhoea, epigastric pain, nausea, vomiting, and weight loss, though many infections are asymptomatic. The diarrhoea can develop into a persistent life threatening type especially in immune-deficient individuals and malnourished children.

Giardia lamblia and *Cryptosporidium* sp., both ubiquitous protozoan parasites, have recently become very important particularly because of their increasing association with drinking water sources (Ayalew *et al.*, 2008; Helmi *et al.*, 2011) and recreational water (Lim *et al.*, 2009; Helmi *et al.*, 2011). Mode of infection of these parasites is basically through a common source, the consumption of water that is contaminated with their cysts or oocysts (Filice, 1952).

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Many countries in the developing world are now faced with inadequate supply of treated water for human consumption. Large populations within both urban and rural areas without access to safe drinking water depend on water from wells, lakes, rivers, streams and other sources which could all be contaminated by external environmental factors (Mons *et al*., 2009; Castro-Hermida *et al*., 2008). In some situations, other animals drink from the same ponds and rivers used by humans. This has consequently increased the risk of water-borne infections, including giardiasis and cryptosporidiosis in many developing countries including Ghana.

As a result of their significant involvement in causing diarrhoeal diseases in impoverished populations and their ability to impair productivity and socio-economic development, giardiasis and cryptosporidiosis have both been categorized among the 'Neglected Diseases Initiative' of the World Healh Organization, in September, 2004 (Savioli *et al*., 2006).

In developing countries in Asia, Africa, and Latin America, approximately 200 million people have symptomatic giardiasis per year and the disease is most prevalent in children less than 5 years old (Shakkoury *et al*., 2005). *Cryptosporidium* is also widespread in the developing world, with 10–30% of individuals being asymptomatic cyst excretors (Current and Garcia, 1991). The frequency of cryptosporidiosis worldwide is often dependent on HIV status. Besides humans, giardiasis and cryptosporidiosis are also common causes of diarrhoeal disease in a number of other mammals (McLauchlin *et al*., 2000; Winkworth *et al*., 2008; Epe *et al*., 2010), which suggests the zoonotic potential of these parasites.

In many countries where diarrhoeal diseases are common, not only have efforts been made by way of research to determine prevalence of these parasites but also currently molecular tools

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are applied to determine the possible sources of contamination of water that could result in human infection (Lim *et al*., 2009; Khan *et al*., 2010). Genotyping provides useful clues to determine sources of infections in outbreak situations, and also clarifies the pathways of zoonotic transmission and host specificity.

1.2 Rationale of study

In Ghana, the fast rate of expansion of new settlements which lack pipe-borne water supply, especially in urban communities, leave large populations to consumption of untreated water. This has consequently increased the risk of water-borne infections, including giardiasis and cryptosporidiosis in the country. Although no major outbreak of these diseases has yet been reported in Ghana, the increasingly high incidence of the diseases reported in our hospitals and polyclinics (24-32%) (Edoh *et al*., 2004; Ayeh-Kumi *et al*., unpub. Adjei *et al*., 2003) as well as in community day-care centres (5.1- 46.5%) (Agyemang, 2006; Atta-Owusu, 2008) suggest that many people are consuming contaminated water. Recent investigations have revealed that some of the sachet water sold for public consumption in Accra contains some enteropathogenic organisms (Kwakye-Nuako *et al*., 2007). Additionally, Dongdem *et al.* (2006) observed that treated water from parts of Accra, contained contaminants including rotaviruses. There is undoubtedly an urgent need to conduct a detailed scientific investigation to ascertain not only safety of drinking water in Ghanaian communities, but also to determine the source of contamination by medically important agents such as *G. lamblia* and *Cryptosporidium sp.*

The (oo)cyst, which is apparently not host specific, has been found in cattle, sheep, rats, mice, cats, dogs, rabbits and guinea pigs (Hamnes *et al*., 2007; Kutz *et al*., 2008). Given that

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in most Ghanaian communities the rearing of domestic animals and keeping of pets in homes are both common practices, thus bringing some animals into close association with human. It is suspected that some level of human infections of the disease could come from infected animals. Presently, the issue of co-infections of diarrhoeal pathogens has also attracted the attention of many investigators worldwide. This has so far not been well investigated in Ghana, where diarrhoeal diseases continue to be the single biggest cause of early childhood mortality with median incidence of diarrhoea of 2.2- 4.7 episodes per child per year (Agbodaze *et al*., 1988). There is thus limited information on the understanding of clinical manifestations associated with co-infections.

Recent reports on diarrhoeal diseases indicate that apart from giardiasis and cryptosporidiosis, rotavirus has become one of the important causative agents in many Ghanaian communities. It has for instance been reported as the predominant cause of acute childhood diarrhoea in urban northern Ghana (Reither *et al*., 2007). Co-infection with *Giardia* could contribute to the severity of the disease. There are reports that a chronic *Giardia* infection could modulate symptoms of rotavirus infection (Fraser *et al*., 2004) when these enteropathogens co-infect a patient. This is worth investigating in Ghanaian communities as rotavirus now represents one of the leading causes of paediatric diarrhoea in the country (Armah *et al*., 2005; 2006; Reither *et al*., 2007).

In Ghana, there is limited information on the identity of *Giardia* and *Cryptosporidium* isolates, their prevalence and impact on public health. A good understanding of the molecular characterization of giardiasis and cryptosporidiosis may be useful in designing strategies to control these diseases, and to help reduce the incidence of diarrhoeal infections, second among the leading causes of infant mortality in Ghana (Afari *et al*., 1988).

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In the present study, isolates of *G. lamblia* and *Cryptosporidium* sp. from children with and without diarrhoea, have been characterized, into genotypes based on variations in the sequence of the glutamate dehydrogenase (GDH) and (18S) subunit of rRNA genes respectively and subsequently by PCR-RFLP and nucleotide sequence analyses. The results provide information on the extent of genetic diversity of *Giardia* and *Cryptosporidium* sp. isolates in the country, and possibility of zoonotic transmission in communities in Accra. Information gathered on clinical manifestations associated with identified genotypes, and co-infections of *G. lamblia* with rotavirus will all be important for clinicians in the country.

1.3 General objective

The main goal is to study the epidemiology and molecular characterization of *Giardia* and *Cryptosporidium* sp. infections among children in Accra, Ghana.

1.4 *Specific objectives*

1. To determine the presence of dual/single infections of *Giardia* and *Cryptosporidium* sp. in children under 5 years
2. To genotype isolates of *Giardia* sp. and *Cryptosporidium* sp.
3. To determine association, if any between genotypes and disease outcome
4. To determine sources of these infections and also clarify the pathways of zoonotic transmission and host specificity from the genotyping data
5. To determine the effect of co-infection of *Giardia* and rotavirus with regards to clinical disease outcome.

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CHAPTER TWO

LITERATURE REVIEW

2.1 Historical background of *Giardia* and *Cryptosporidium* parasites

Giardia was first identified in human stool by Antonie van Leeuwenhoek in 1681 (Boreham *et al*., 1990). However, it was not recognized as a human pathogen until the 1960s, after community outbreaks and its identification in travellers (Craun, 1986; Farthing, 1992). *Cryptosporidium* was also first recognized as a potential human pathogen in 1976 in a previously healthy 3-year old child (Nime *et al*., 1976). A second case of cryptosporidiosis was reported two months later in an individual who was immunosuppressed as a result of drug therapy (Meisel *et al*., 1976). The disease became best known in immunosuppressed individuals exhibiting the symptoms now referred to as acquired immunodeficiency syndrome, or AIDS (Hunter & Nichols, 2002). The parasite was first described by Tyzzer (1907), when he isolated the organism, which he named *Cryptosporidium muris*, from the gastric glands of mice. Tyzzer (1912) found a second isolate, which he named *C. parvum*, in the intestine of the same species of mice.

2.2 Morphology and classification of *Giardia* and *Cryptosporidium*

Cryptosporidium and *Giardia* are genera of protozoan parasites. There is lack of morphological characteristics to distinguish between parasites of the same species but which vary at the genetic level. The advent of molecular tools and their application to medical research however, has helped in clearing some of the controversies that have surrounded the taxonomy and classification of these two organisms in the past.

G. lamblia is a flagellated protozoan parasite (Phylum Protozoa, Subphylum Sarcomastigophora, Superclass Mastigophora, Class Zoomastigophora, Order

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Diplomonadida, Family Hexamitidae). The taxonomy of *Giardia* at the species level is complicated and unresolved because of limited morphologic differences. Based on morphology, six species of this genus are considered valid. These include *G. lamblia* (syn. *G. duodenalis* or *G. intestinalis*) in a wide range of mammals, including humans, livestock, and companion animals, *G. agilis* in amphibians, *G. muris* in rodents, *G. ardeae* and *G. psittaci* in birds, and *G. microti* in muskrats and voles (Filice, 1952; Erlandsen *et al.*, 1990; van Keulen *et al.*, 1998; Robertson *et al.*, 2007). However, on the basis of host origins, 41 *Giardia* species have been named (Van Keulen *et al.*, 1993).

Cryptosporidium is a protozoan parasite (Phylum Apicomplexa, Class Sporozoasida, Subclass Coccodiasina, Order Eicoccidiorida, Suborder Eimeriorina, Family Cryptosporidiidae). The genus *Cryptosporidium* now comprises 14 species, namely *C. hominis* in humans and monkeys, *C. parvum* in cattle, other mammals, and humans, *C. andersoni* in cattle, *C. muris* in rodents, *C. suis* in pigs, *C. felis* in cats, *C. canis* in dogs, *C. wrairi* in guinea pigs, *C. bailey* in poultry, *C. meleagrides* in turkeys and humans, *C. galli* in finches and chicken, *C. serpentis* in reptiles, *C. saurophilum* in lizard, and *C. molnari* in fish (Xiao, *et al*. 2004).

2.3 Morphology of *Giardia* and *Cryptosporidium*

Giardia has two distinct morphological forms, namely trophozoite (feeding or proliferative form) (Fig. 1a) and cyst (resting or infective form) (Fig. 1b). The trophozoite, or feeding stage, lives mainly in the duodenum but is often found in the jejunum and ileum of the small intestine. Trophozoites (9–21 μm long, 5–15 μm wide and 2–4 μm thick) have a pear shaped body with a broadly rounded anterior end, two nuclei, two slender median rods, eight flagella in four pairs, a pair of darkly staining median bodies and a large ventral sucking disc

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(cytostome). Trophozoites are normally attached to the surface of the intestinal villi, where they are believed to feed primarily upon mucosal secretions. After detachment, the binucleate trophozoites form cysts (encyst) and divide within the original cyst, so that four nuclei become visible. Cysts are ovoid, 8–14 μm long by 7–10 μm wide, with two or four nuclei and visible remnants of organelles. Environmentally stable cysts are passed out in the faeces, often in large numbers.

The various stages or forms of *Cryptosporidium* parasite are, the oocysts, sporozoites, and merozoites. The round to oval oocyst, are 4-6 μm in diameter. They seem often refractile at wet smear. Black dot or small vacuoles in oocyst could be seen after modified acid fast staining. Sporozoite and merozoites have apical complex (microneme, rhoptry, conoid, preconoidal ring) at the anterior most part.

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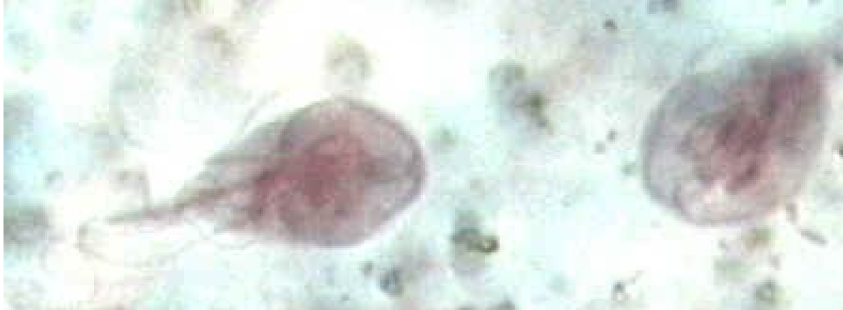

Fig. 1a: *Giardia lamblia* trophozoites trichrome stained from a duodenal aspirate. The nuclei and the flagella are clearly visible. The adhesive disc and 4 posteriorly directed flagella are visible in the trophozoite on the left hand side. **Source: (www.dpd.cdc.gov)**

Fig. 1b: *Giardia lamblia* cysts iodine stained. They are oval in shape often showing their four nuclei. The filbrils and flagella lie longitudinally along the body. **Source: (www.cdfound.to.it)**

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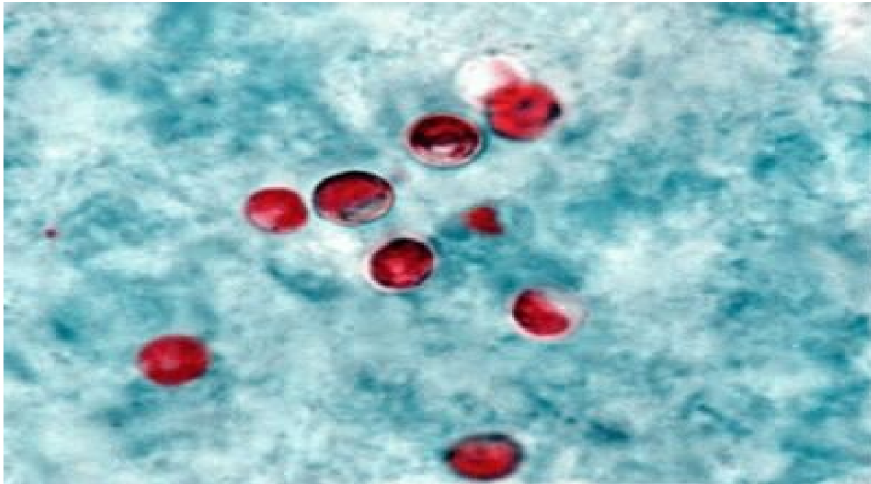

Fig. 1c. *Cryptosporidium* oocysts. Numerous pink-red-stained oocysts. Modified Kinyuon acid-fast stain (40X). **Source: http//labmed.ascpjournals.org/content/39/4/23**

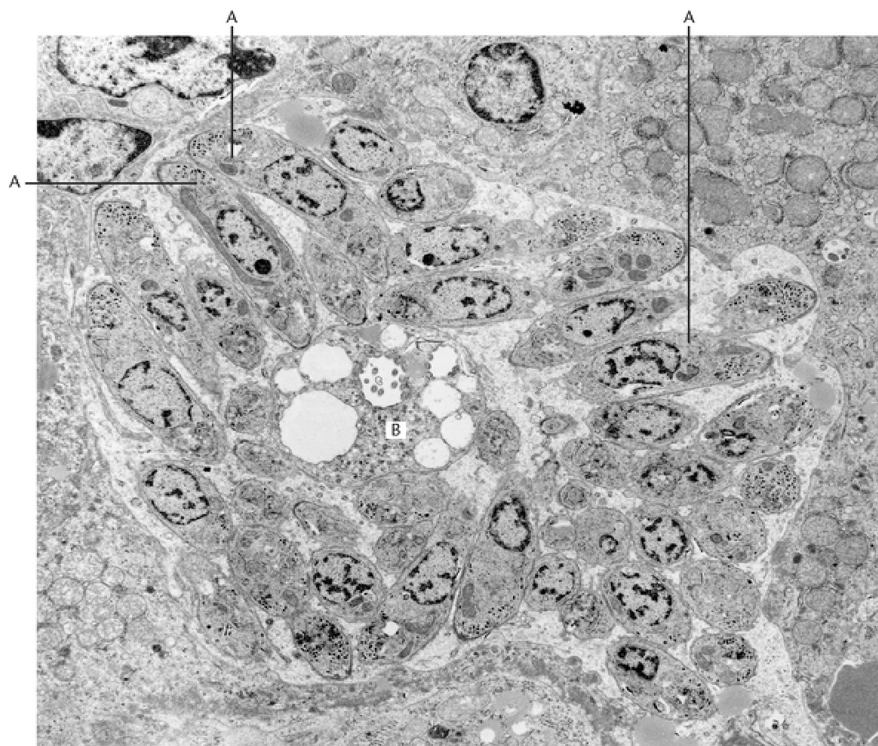

Fig. 1d. *Cryptosporidium* Merozoites that have completed budding from (B), centrally positined. **Source: http//www.els.net/WileyCDA/ElsArticle/refld**

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2.7 Genetic variability and subtypes (genotypes) of *Giardia and Cryptosporidium*

G. lamblia has six distinct genotypes, namely assemblages A and B found in humans and other mammals, C/D in dogs, E in livestock, F in cats, and G in rats (Amar *et al*., 2002; Sulaiman *et al*., 2003). Subgenotyping techniques have further classified assemblage A into two genetic groups, A-I and A-II, genotype B into BIII and BIV (Abe and Teramoto, 2011). Type A-II is exclusive to humans, while type A-I occurs in humans, dogs, rodents, and other animals but not cattle (Sulaiman *et al*., 2003; Monis *et al*., 2003;). *G. lamblia* isolates from both domestic animals and humans have been characterized based on variations in the triosephosphate isomerase (TPI) and glutamate dehydrogenase GDH genes, and the small subunit ribosomal RNA (SSU rRNA) (Sulaiman *et al*., 2003; Abe *et al*., 2005; Wielinger and Thompson, 2007). Other amplification targets for *Giardia* include, β-giardin, EF-1α and GLORF-C4 (Caccio *et al*., 2005). Genotyping provides useful clues to determine sources of infections in outbreak situations, and also clarifies the pathways of zoonotic transmission and host specificity.

With regards to *Cryptosporidium*, humans are most frequently infected with *C. hominis* and *C. parvum* (Cama *et al*., 2008; Pelayo *et al*., 2008; Yakoob *et al*., 2010; Sulaiman *et al*., 2005). Molecular characterization of the 60-kDa glycoprotein (GP60) gene of *C. hominis* and *C. parvum* has enabled further division into subtype families and subtypes (Sulaiman *et al*., 2005). Molecular analyses indicate that *C. parvum* which is the major cause of cryptosporidiosis in humans comprises of at least two different genotypes (Cama *et al*., 2008). These are genotypes 1 (or human type), and genotype 2 (or calf type). Whilst genotype 1 is restricted to humans, genotype 2 is found in livestock as well as humans.

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Anthropophilic and zoonotic species such as *C. meleagridis* in turkeys, *C. muris* in mice, and *C. felis* in cats have also all been implicated in human illness (Morgan *et al.*,1998).

Identification of *C. parvum* genotypes has also been achieved through the amplification of the *Cryptosporidium* oocyst wall protein (COWP) (McLauchlin *et al.*,2000). Other *Cryptosporidium* gene targets for amplification include the 18SrDNA, Hsp70, Actin, β-Tubulin, GP60, Microsatellites, Minisatellites, and Extrachromosomal double-stranded RNA (Caccio *et al.,* 2005).

2.5 Epidemiology of *G. lamblia* and *Cryptosporidium* sp. infections

The epidemiology of *G. lamblia* and *Cryptosporidium* sp infections has been well documented in many countries worldwide especially where the diseases are common, and increasingly pose a threat to the health of children. Generally, the prevalence of these enteric parasites are higher in developing countries as compared with developed nations (Gelanew *et al.*, 2007; Pinheiro *et al.,* 2011; Friesema *et al.*, 2011), except in disease outbreak situations such as occur during contamination of domestic water supply systems (Castro-Hermida *et al.*, 2008; Robertson *et al.*, 2007). Individuals working in dairy farms in developed countries also have the risk of zoonotic transmission, and occasionally become infected (Khan *et al.*, 2010; Hsu *et al.*, 2007; Mark-Carew *et al*; 2010). The scope in knowledge of prevalence, disease burden, and risk factors for infection continue to broaden, due to renewed interest in waterborne infections and the recent application of molecular tools in studying these diseases.

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2.5.1 Prevalence of *G. lamblia* and *Cryptosporidium* sp. infections

2.5.1.1 Asymptomatic Infections

Giardiasis and cryptosporidiosis are common among children in daycare centres (Al Braiken *et al.,* 2003; Gelanew *et al.*,2007), nursery and primary schools (Al-Saeed and Issa, 2010; Perez-Cordon *et al*., 2008; Ratanapo *et al*., 2008), orphanages, and other social institutions (Suwan *et al*., 1992), as well as urban slums (Mehraj *et al*., 2008), usually reported as asymptomatic infections. Determination of the prevalence of asymptomatic giardiasis and cryptosporidiosis in social institutions and daycare centers is important as it draws the attention of health authorities to the public health risk within such institutions. In each of all 10 schools selected randomly for a study at western Tajikistan, in Asia, children were noted to be infected with *G. lamblia*, with a prevalence of 26.4% (Mathys *et al*., 2011). Similarly, among children of some three marginal urban districts of Trujillo (Peru) *G. lamblia* was identified as the most frequent parasite with a prevalence rate of 23.8%, whilst prevalence of *Cryptosporidium* spp was 2% (Perez Cordon *et al*., 2008).

Asymptomatic infections of both parasites are also common in many African countries especially in daycare centers, and pre-schools ranging between 5% -49% (Al-Hindi and El-Kichaoi, 2008; Eyasu *et al*., 2010). Different rates of asymptomatic infections have been identified in the Ghanaian population. Low prevalence rates of 9.7% and 0.8% for *G. lamblia* and *Cryptosporidium* sp. respectively were recorded among 124 children used as controls in a study at Northern Ghana (Reither et al., 2007). However, higher prevalence rates of *G. lamblia*, ranging from 5.1- 46.5% have also been reported ((Agyemang, 2006; Atta-Owusu, 2008; Anim-Baidoo *et al*., *in press*). Very little information exists generally for asymptomatic *Cryptosporidium* sp infection among children in Ghana. Adjei *et al*. (2004)

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recorded a prevalence rate of 15.6% asymptomatic cryptosporidiosis, and a relatively higher rate (27.8%) among same age group of children with diarrhoea in Accra. There are also few reports available on its prevalence in HIV patients on admission at the Korle Bu Teaching Hospital at Accra (Adjei *et al*., 2003), showing the opportunistic nature of this entero-pathogen. Generally, the parasite tends to have a higher prevalence among individuals with compromised immune systems of the body.

2.5.1.2 Symptomatic Infections

Symptomatic cases of *G. lamblia* and *C. parvum* infections have been reported from children hospitalized for acute diarrhoea worldwide, and different prevalence rates have been recorded. Comparatively, prevalence of symptomatic giardiasis in most reports has been lower than asymptomatic giardiasis. In a retrospective study conducted in 2007 at the Academic Paediatric hospital, 'Centro Havana', (APHCH), Havana, Cuba, medical records revealed that a total of 185 children were hospitalized for giardiasis at that hospital for the year 2007 (Escobedo *et al*., 2011). Clinical information which accompanied the data also indicated that the mean length of hospital stay was 4.9 days. The study provided an opportunity for the clinicians to observe the clinical significance of symptomatic giardiasis, and also obtain information on prevalence of hospitalized cases, which according to the authors was limited in Cuba. The study did not however provide any information on cryptosporidiosis. In an earlier study however, *Cryptosporidium* sp. was reported as an important cause of diarrhoea among Cuban children (Pelayo *et al*., 2008). A prevalence rate of 26.8% giardiasis has been reported among Cuban children hospitalized for acute diarrhoea (Bello *et al*., 2011).

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A hospital-based surveillance of enteric parasites at Kolkata, India revealed that both parasites were associated with diarrhoea in the area (Mukherjee *et al*., 2009). The study was conducted among children admitted to the Infectious Diseases (ID) hospital at Kolkata between the periods of November, 2007 and October, 2008 for complaints of diarrhoea. Until the study was carried out, the burden of enteric parasitic infestations in and around Kolkata was not reported. The authors identified a prevalence rate of 13.3% for *G. lamblia* infections, and 7.6% for *Cryptosporidium* sp. Through this study, the areas in and around Kolkata where infections were high or low were all identified. They reported that Rajarhat, Tangra and Tiljala areas of Kolkata had the highest incidence of giardiasis followed by Beliaghata and Salt Lake city. For cryptosporidiosis, Salt Lake city, Dum dum, Tangra and Narkeldanga had the highest incidence followed by Rajarhat and Phoolbagan. The study established that, *Cryptosporidium* sp. and *G. lamblia* were both potential causes of severe diarrhoea in Kolkata, India.

At the gastro-enterology outpatient clinic of Aga Khan University (AKU) hospital in Karachi, Pakistan, patients screened for both *G. lamblia* and *Cryptosporidium* infections by microscopy and polymerase chain reaction (PCR) showed that the infections were common (Yakoob *et al*., 2010). Prevalences were 6.3% and 8.7% by microscopy and PCR respectively, for *G. lamblia*, whilst *Cryptosporidium* had prevalences of 3.9% and 42% respectively. It was identified that *Cryptosporidium* was significantly associated with chronic diarrhoea.

In comparing prevalence of both parasites in Saudi children with and without diarrhoea, it was revealed that, out of 63 diarrhoeal stool samples collected from four paediatric clinics, infection was higher for *Cryptosporidium* (32%), compared with *G. lamblia* (29%) (Braiken

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et al., 2003). The observations made raised issues of public health importance as the authors indicated that previous studies in the same area had reported a considerably lower rate of *Cryptosporidium* infection. This implied that there had been a higher level of exposure to *Cryptosporidium* infections over the years. In Iran, 25.6% prevalence of *Cryptosporidium* infections was reported for patients with diarrhoea (Mirzaei, 2007). Although the study covered a wide range of age groups including adults, it showed that *Cryptosporidium* infection was a paediatric health problem in Iran. The study however did not consider *G. lamblia* infections.

In several studies conducted in African countries, varying rates of prevalence for *G. lamblia* and *Cryptosporidium* sp. have been reported. In most of these studies however, the prevalence rates could have been underestimated as microscopy which has a lower detection rate have been used. In Lagos, Nigeria, a lot more children (≤ 5 years) report more of cryptosporidiosis than giardiasis at the hospital with prevalence of 27.6% and 4.8% respectively (Wellington *et al*., 2009). These parasites are also common in Kaduna state of Nigeria (Makai *et al*., 2012), where modern molecular techniques were applied to detect infection rate. In that study, the infection rates reported were low, 1.9% and 3.2% for cryptosporidiosis and giardiasis respectively. These parasites have also been found among children from the Cufada lagoon natural park at Guinea-Bissau (Ferreira *et al*., 2012), where through genotyping techniques, the source of infection was traced.

Mandomando *et al.* (2007) reported similar low prevalence of *G. lamblia* (2.5%) in Mozambique children hospitalized for diarrhoea. Also, in the Chobe district of Botswana, both *Cryptosporidium* sp. and *G. lamblia* were identified to be contributing significantly to recurrent diarrhoeal cases (Alexander *et al*., 2012). Reports from that study also indicate that

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Cryptosporidium sp. appeared to be potentially a cause of diarrhoeal disease among children in the area. Children of age 2 years and below were significantly affected by the parasite than any other age group. The lack of sufficiently well treated water for consumption has resulted in high prevalence rates in some African countries. For instance, in the Northwestern Ethiopian town of Pawi, high prevalence rates of 26.6% and 81% *G. lamblia* and *Cryptosporidium* sp were attributed predominantly to the lack of potable water (Eyasu *et al.*, 2010). A study on water supply and distribution system in Addis Ababa, Ethiopia, showed that water from treated water storage tanks, and tap water for public consumption were all contaminated with *G. lamblia* and *Cryptosporidium* oo(cysts) (Tesfalem *et al.*, 2012).

In Ghana, giardiasis and cryptosporidiosis have both been reported in several studies throughout the country. The prevalence of these parasites among diarrhoeic children in northern Ghana were comparatively low, 9% for *G. lamblia* and 0.4% for *Cryptosporidium* sp. (Reither *et al.*, 2007). Nkrumah and Nguah (2011) used hospital records in a retrospective study to assess the involvement of intestinal parasitic infections in acute diarrhoea cases among children at Agogo, located in the mid-belt of Ghana. These were records of children less than 18 years whose stool samples were presented to the laboratory of the Agogo Presbyterian hospital for parasitological test, between the periods of January, 2006 and May, 2009. The patients were referred from the Child Welfare Clinic (CWC), paediatric out-patient clinic and Children's ward of the hospital. From the study, *G. lamblia* (9.7%) was identified as the most prevalent intestinal parasite in the area, which was also associated with unhygienic practices. The prevalence of *Cryptosporidium* sp. was not reported.

A recent study involving the aetiology of paediatric diarrhoea in southern Ghana (Opintan *et al*, 2010), revealed that *Cryptosporidium* was rather the most common enteroparasitic agent

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in Accra. They reported a prevalence of 8.7% in their study which was conducted at the Princess Marie Louis Children's Hospital (PML), a major children's hospital in Accra, Ghana. *G. lamblia* infection was absent among the children studied. In another hospital-based study conducted at the Korle-bu Polyclinic, also located in Accra, the prevalence of *G. lamblia* was shown as 10.1% (Anim-Baidoo *et al*., *in press*). In this study, children of age 5 years and below who had been hospitalized for acute diarrhoea were screened for *G. lamblia* infections. A major limitation of this study was the short duration within which it was conducted, for which reason a large sample size could not be obtained. In spite of this limitation, reports from the study support previous observations made by other investigators that *G. lamblia* contributes to diarrhoeal cases among children in Accra.

2.5.2 Risk factors for *G. lamblia* and *Cryptosporidium* sp. infections

A number of factors have been found to be associated with the incidence of giardiasis and cryptosporidiosis. The identification of these factors enable health authorities to initiate preventive and control measures, in order to reduce incidence within communities in which the diseases are endemic. Examples of such risk factors which generally vary from one population to the other include age, sex, geographical location, season, family history, breastfeeding habits, some domestic and social settings, as well as environmental and zoonotic factors (Pereira *et al.,* 2007; Fraser *et al*., 2000; Stuart *et al*., 2003).

2.5.2.1 Age, sex, geographical location, and seasonal variations

There are seemingly conflicting reports on the nature of association that exists between gender (sex), age, geographical location of child, or seasonal variations and the incidence of cryptosporidiosis and giardiasis among children worldwide. Among Brazilian children hospitalized for diarrhoea, Pereira *et al* (2007) observed that age of child was positively

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associated with the odds of *G. lamblia* infection; and that the odds of giardiasis increased about 1.18 for each additional year of age (OR, 1.18; 90% CI, 1.0-1.36; P=0.052). The majority of infected children were between 24-48 months of age (19.7%). Sex and weight however were not found to be associated with giardiasis in their population of study (P>0.10). Season was also not associated with odds of *G. lamblia* infection. Among Brazillian children from a daycare center in the region of Presidente Prudente, Sao Paulo, Brazil however, there was higher prevalence of giardiasis in boys than girls (Tashima *et al*., 2009).

In contrast to observations made by Pereira *et al.* (2007), but in support of Tashima *et al.* (2009), significantly more giardiasis cases identified in Cuban children were boys than girls (P<0.01) (Escobedo *et al*., 2010). This difference was attributed to gender-associated differences in exposure to *Giardia*. In other words, the behaviour and recreational exposure of boys differ from those of girls in some settings. Infection occurred most among children of age 1-4 years. Similar reports were made by Hussein (2010) among Iraqi children on admission at the Maternity and children teaching hospital in Thi-Qar, Southern Iraq. Reports from this study also indicated that infection was significantly higher in children living in rural areas than those living in urban area (28.2%, 15.2%, P< 0.05). Additionally, rate of infection during hot season was higher than the cold season (69.1% vs 30.8%). This could be due to the fact that, cold weather kills the infective cysts (Bingham and Meyer, 1979).

Other behavioural factors that could be involved include greater consumption of water and drink in hot weather which may be sources of infection. Bello *et al.* (2011) reported that Cuban children aged 5 years and over, appeared to be at greater risk of *G. lamblia* infection than the younger ones, but gender had no marked effect on risk. Children who lived in a rural

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area appeared to be at a 3.1-fold greater risk of *Giardia* infection (OR= 3.01; CI=1.23-7.35). In Kolkata, India, the age group >5-10 years was predominantly infected with *G. lamblia* (p= 0.001: OR= 3.937; 95% CI=1.862-8.326) (Mukherjee *et al.,* 2009), which was in support of recent reports by Bello *et al.* (2011) . Regarding seasonality, Mukherjee *et al.* (2009) observed that whilst the occurrence of *G. lamblia* remained almost unchanged throughout the year, the occurrence of *Cryptosporidium* sp showed differential seasonal distribution. In a rural community of Thailand, children of age 5- 9 years old were reported to have the greatest risk (1.3 times greater) of getting infected with giardiasis (Ratanapo *et al*., 2008).

Cryptosporidium infections have been reported to be most common among infants of age 2 years and below (Lindo *et al*., 1998; Suwan *et al*., 1992; Bogaerts *et al*., 1984). However, in an epidemiological investigation of cryptosporidiosis in Cuban children, it was observed that there was no gender or age difference (Pelayo *et al*., 2008). The inclusion of children more than 5 years in the study (5-8 years) indicated that, in Cuba, cryptosporidiosis is a disease not only of infants but also of children of school age.

Generally, information obtained on risk factors for giardiasis and cryptosporidiosis in Africa show similar trends with those reported from other developing nations worldwide. In a seven year (1991-1997) community-based studies in Guinea-Bissau, West Africa among children of age 5 years and below with diarrhoea, children aged 6-11 months had the highest risk of infection with *Cryptosporidium* (Perch *et al*., 2001). In this study, whilst there was marked seasonal variation with cryptosporidiosis infections (peak of infection period just before rainy season), giardiasis had no seasonality. Similar studies in Southern Guinea-Bissau indicate that prevalence of cryptosporidiosis was highest in children of age 7-12 months

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(Carstensen *et al*., 1987). In Kwara state, Nigeria, cryptosporidiosis was most common among children of age less than 2 years in a study in which children of age ≤14 years with diarrhoea were screened for intestinal parasites (Nwabuisi, 2001). In Lagos, Nigeria, the age with highest infection was 4-5 years old ($P < 0.001$) for both giardiasis and cryptosporidiosis (Wellington *et al*., 2009). In another African country of Ethiopia, significantly higher prevalence of giardiasis occurred among females than males, but no association was observed for cryptosporidiosis infection between the two sexes (Eyasu *et al*., 2010). Additionally, prevalence of *G. lamblia* and *C. parvum* infections were not significantly different among the age groups. Similarly, *G. lamblia* infections was reported among all age groups of children (mean = 10.68 years) studied in Botswana (Alexander *et al*., 2012), but the study also indicates that *C. parvum* infection was predominantly higher in children less than 2 years.

Seasonality significantly influences transmission of both cryptosporidiosis and giardiasis among children in the city of Kafue, Zambia where there were more infections in the wet compared to the dry season (34.8%, 162/466 vs. 24.7%, 79/320, $P = 0.003$ and 35.2%, 164/466 vs. 20.0%, 64/320, $P < 0.001$, respectively) (Siwila *et al.,* 2011). Apart from reporting that both parasites were common in the African country of Zambia, their study also indicates that diarrhoea was significantly associated with cryptosporidiosis (RR = 1.23, 95% CI = 1.03-1.47; $P = 0.029$) but not with giardiasis (RR = 1.12, 95% CI = 0.91-1.53; $P = 0.26$).

Wongstitwilairoong *et al*. (2007) observed that high temperatures in combination with high rainfall were conducive to acquiring intestinal parasites among pre-school children in Sangkhlaburi, Thailand. In their study they reported that *G. lamblia* and *Cryptosporidium* infections peaked during rainy season, and the lowest percentage occurred during hot season.

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In Kenya, cryptosporidiosis was reported to be most common among children of age 13- 24 months and least among 48- 60 months of age (Wangeci *et al*., 2006). The authors indicated that infection was highest in November to February, which coincided with the hot and dry season. It was explained that during the hot season there is usually shortage of water, which makes people look for water from all sources including wells, known to harbour oocysts of the parasite. They recommended further studies on molecular investigation of the *Cryptosporidium* species and subtypes in circulation in order to have a full understanding of the transmission dynamics in the area. In a hospital-based study in Accra, Ghana, *Cryptosporidium* sp. infection was highest among children of ages between 6 and 24 months (Adjei *et al*., 2004) which agrees with most of the studies reported in other developing countries worldwide.

2.5.2.2 Overcrowding, family history, and faecal disposal at home.

Homes overcrowded with people is a common phenomenon in most developing countries for socioeconomic and cultural reasons. Unfortunately, the situation could promote high level of inter personal transmission of infectious diseases. In overcrowded homes, the level of sanitation could be deteriorated especially where children are not well monitored or taken care of in the household. Sanitation level could worsen in overcrowded homes that lack modern or adequate toilet facilities, as occurs in many rural communities where defaecation occur in the open area leading to high levels of contamination of the environment. Giardiasis and cryptosporidiosis have a common source of infection, which is through the consumption of water contaminated by cysts and oocysts of the parasites (Eyasu *et al*., 2010; Ayalew *et al*., 2008).

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In studying children suffering from acute diarrhoea in Iraq, it was revealed that, there was a higher prevalence of giardiasis for households with four or more children (28.1%) compared to households with fewer children (18.3%) (Hussein, 2010). In another study, Brazilian children who lived in a family with other young children (<10 years old) were at greater risk for *G. lamblia* infections compared to children in families without additional young children (Pereira *et al*.,2007). Also, in a rural community of Thailand, having more than 3 children per household of an age under 12 years old increased by 2.5-fold the risk of contracting *Giardia* (Ratanapo *et al*., 2008). This study showed that person-to-person transmission is also an important transmission pathway of giardiasis in that rural community. In contrast to many reports, a 2-year birth cohort study among Bedouin infants revealed that there was no association with number of children aged ≤ 5years in the household and carriage of *G. lamblia* infections (Frazer *et al*.,2000). Through a hospital-based study, Bello *et al*. (2011) observed that, in Cuba, children who already had a personal history of parasitic infection had a 23% increased likehood of having a current *Giardia* infection. Those who had a family history of parasitic infection had a 96% increased likelihood of current *Giardia* infection.

2.5.2.3 Daycare Centres and Orphanages

Institutionalization is one of the risk factors for intestinal protozoan infection including giardiasis and cryptosporidiosis among children (Suwan *et al*.,1992). The activities of daycare centres throughout the world continue to gain much recognition and patronage as a result of the roles they play in supporting many parents for the upkeep of their children. Generally, children who attend daycare centres spend a considerable length of time each day at the centre under the care of attendants who are supposed to be well trained to handle children. Orphanages throughout the world continue to serve as permanent homes for children without parents or guardians. In such institutions however, there is generally close

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physical contact between the young children which could lead to easy spread of infections. Coupled with poor personal hygiene, the risk of infection increases within daycare centres and orphanages. Many of the infections usually occur as asymptomatic (Termmathurapoj *et al*; 2000) but there are symptomatic cases also (Pelayo *et al*., 2008). Infections could also come from the care givers or attendants at such institutions (Mahdi *et al*., 2002).

An epidemiological report on children admitted at clinics and hospitals in Cuba for acute cryptosporidial diarrhoea (Pelayo *et al*., 2008) revealed that most of the children attended primary schools (15 out of 28), or day-care centres/kindergarten (12 out of 28), but one child, aged 2 years was cared for at home. This study shows that children catered for at home are at much lesser risk of *Cryptosporidium* infection than those in daycare centres. Among orphans in the Chiangmai Reception Home, Chiang Mai, both parasites were common, with *Giardia* infections (35.8%) being higher than *Cryptosporidium* infections (5.9%) (Suwan *et al*.,1992). A significant observation made in this study was that none of the babies (1-5 months) was infected. This was attributed to the provision of well treated drinking water (boiled and filtered) as well as well cooked food for the babies. Infection was rather high among the toddlers (6 months -2 years), which the investigators in this study explained could be due to oral-faecal transmission. They pointed out that higher *Giardia* infection rate observed in the study could also be due to lower infective dosage of cysts needed to initiate infection.

Several studies conducted in orphanages and daycare centres in Accra, Ghana (Atta-Owusu, 2008; Agyemang, 2006; Anim-Baidoo *et al*., *in press*) have shown that both *Giardia* and *Cryptosporidium* are common parasites responsible for asymptomatic infections in children who patronize these institutions. All the children in these studies appeared healthy without

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any noticeable clinical symptoms. In all these studies, unhygienic practices and poor environmental sanitation were suspected to play a role in transmission of the parasites.

2.5.2.4 Source of water and food for consumption

The supply of water to a community is an important risk factor for giardiasis and cryptosporidiosis (Isaac-Renton *et al.*, 1999). Several outbreaks have resulted from the contamination of municipal water supplies with *Giardia* cysts and *Cryptosporidium* oocysts (U.S. EPA, 1998; Robertson *et al.*, 2007). This is partly because, the normal chlorine level used to kill bacteria in municipal water supplies will not inactivate *Giardia* cysts or *Cryptosporidium* oocysts (Castro-Hermida *et al.*, 2008). The sources of water to people in communities vary from one community to the other, and examples include pipe –borne water, wells, rivers, boreholes, and sachet water (bagged water). They are also categorized into protected (springs, boreholes, as well as deep and shallow protected wells) and unprotected (surface water and rivers) water sources. Many studies conducted on surface waters have revealed contamination with *Cryptosporidium* sp. and *G. lamblia* (Carmena *et al.*,2008; Monis *et al.*, 2008; Lim *et al.*, 2009; Helmi *et al.*, 2011). This essentially suggests that treatment processes must be regularly monitored to ensure that water is properly disinfected before supplied to the public for consumption.

Ayalew *et al.* (2008) screened Ethiopian (Lege Dini) children for *Cryptosporidium* and *G. lamblia* infections to ascertain whether infection correlates with their source of drinking water. There were two major sources of water namely, protected and unprotected sources. They observed that there was no difference in infection between children drinking water from protected and unprotected sources ($p > 0.05$). To explain why the different water sources did not correlate with infection in the children, the authors suggested that other factors may have

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contributed to infection. These factors include water storage conditions at home, absence of sanitary facilities, observed indiscriminate defaecation by the inhabitants and their animals, living in crowded situations, poor personal hygiene, and high illiteracy rates.

In another study which was carried out in selected villages of Pawi Special District in Benishangul-Gumuz region, Northwestern Ethiopia, Eyasu *et al.* (2010) screened children who were drinking water from different sources, for *Cryptosporidium* and *Giardia* infections. In their study, it was revealed that infections with the two parasites in the children was associated with source of drinking water. There were more cases of giardiasis detected in children who depend on water from unprotected source. On the other hand, more cases of cryptosporidiosis were detected in children who used protected water source. The different water sources were Ali-spring (unprotected), Diga dam (unprotected), Hand-pump (protected), and manually dug well (unprotected). They explained that some of the sources, such as Ali-spring and Diga dam were highly exposed to run off during the rainy seasons. Through the process, faecal matter from both human and animals are washed into the main water source, and this could be a good source of contamination of the water with cysts and oocysts of the parasites. Higher prevalence of *Cryptosporidium* in protected water source was explained to be as a result of inadequate protection from sources of contamination, as well as unhygienic use of water in the house. In Cuba, Bello *et al.* (2011) observed that children from households that did not receive water from an aqueduct were at relatively high risk of *Giardia* infection as were those who drank unboiled tap water. In Thailand, drinking bottled water was identified as a risk factor for giardiasis among Primary school children of a rural community (Ratanapo *et al.*, 2008).

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Among Iraqi children with acute diarrhoea, Hussein (2010) identified highest rate of *G. lamblia* infection in children who drank raw (untreated) water (62.5%), followed by municipal water (36.3%), RO or purchased bottled water (6.1%). The author explained that the high prevalence may be due to contamination of municipal water supplies with human waste, poor quality of water, faulty of sewage line, and insufficient level of chlorine.

Until recently, the main sources of drinking water in many communities in Ghana were, pipe-borne, boreholes, wells, streams and rivers. The introduction of sachet water (bagged water) onto the Ghanaian market now has received a high degree of patronage by the general public. This is because it is considered to be safe, hygienic and affordable. However, the purity and safety of the sachet water has been challenged by some researchers in Ghana. Kwakye-Nuako *et al.* (2007) screened some twenty seven different brands of sachet water samples obtained from vendors in Accra, for parasitic agents. They reported that seventy-seven percent (77%) of the samples contained infective stages of various pathogenic parasitic organisms including oocysts of *Cryptosporidium*. Reasons advanced by the authors of this study to possibly explain their observations include improper processing and purification procedures, unhygienic handling after production, the small size of the pathogens which enable them to escape filtration, and the resistance of these pathogens to physical water treatment agents and disinfectants. The findings of the study is very crucial as it suggests the need for regular testing and monitoring of sachet water sold in the city of Accra, Ghana.

Many communities in Kumasi, Ghana depend on wells and borehole water for drinking. Unfortunately, a sanitation survey around the wells and boreholes conducted by Obiri-Danso *et al.* (2008) indicates that these water sources were frequently sited near latrines, refuse tips and other social amenities, and in the vicinity of domestic or grazing animals.

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Their study also revealed that most of the wells that the local communities depend on are contaminated with faecal matter. This is also likely to be a source of *Cryptosporidium* and *Giardia* infections in the community.

Unhygienic food handling procedures can also lead to contamination of food by the cysts and oocysts of *G. lamblia* and *Cryptosporidium* sp. respectively. The cysts and oocysts are very small in size, and environmentally robust. Additionally, very high numbers are shed by infected individuals, with very low dosage required to initiate human infection. Vegetables and fruits may be contaminated with cysts or oocysts in the field during chain irrigation and fertilization activities. Pereira *et al*. (2007) observed that the consumption of raw salad food, cabbage was associated with a 2.9 times greater odds of *G. lamblia* infection in Brazilian children with diarrhoea, compared to the odds of infection for similar children whose diet did not include this salad item. The consumption of milk however was found not to be associated with *G. lamblia* infection in this study.

In a retrospective study involving diarrhoeal patients in a district hospital in Ghana, Nkrumah and Nguah (2011) identified that the buying of food from street vendors, some of whom do not practice proper personal hygiene as one of the main contributing risk factors associated with *G. lamblia* infections.

2.5.2.5 Breastfeeding habits

Several studies conducted on breastfeeding habits attest to the many benefits obtained from breastfeeding with regards to infant health, as well as intellectual and motor development (Mortenson *et al*., 2002; Horwood *et al*., 2001). An infant may be breastfed exclusively, partially (predominantly), or not breastfed at all depending, in most cases, on the choice of

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the mother. By definition, exclusively breastfed child is the one who received no solids, non-breastmilk or water, or other liquids for 3 – 6 months. On the other hand, predominantly or partial breastfed child is the one who received solids, non-breast milk, juices, water, teas, and other liquids (Kramer *et al*., 2001; Betran *et al*., 2001). There are also reports that both morbidity and mortality rates are lower in breastfed than in nonbreastfed infants due to immunological and nutrional protection that breast milk provides for the infant (Arifeen *et al*., 2001; Betran *et al*., 2001; Field, 2005). Exclusive breastfeeding has been reported to be more protective than partial breastfeeding (Molback *et al*., 1997), and the World Health Organization has recommended a period of first six months of the baby for an exclusive breastfeeding (Kramer and Kakuma, 2004).

Protection offered against *G. lamblia* infection by breastfeeding was reported over two decades ago, among Mexican infants (Morrow *et al*., 1992). In a prospective birth cohort study, the authors reported that compared with exclusively breastfed infants, partially breastfed infants had a risk ratio of 3, whilst infants who were not breastfed a risk ratio of 5. Pereira *et al*. (2007) on the contrary observed that among Brazilian children who had been hospitalized for diarrhoea, children of 0.05- 1.7 years of age who had been breastfed did not have a lower risk of *G. lamblia* infection compared to non-breastfed diarrhoeic children within the same age group. In a one-year birth cohort study to investigate the impact of breastfeeding on *G. lamblia* infections in Bilbeis, a rural community in Egypt (Mahmud *et al*., 2001), it was observed that exclusively breastfed infants had lower risk for asymptomatic (odds ratio [OR] =0.66, 95% [CI]=0.45- 0.96, P< 0.05) than symptomatic infections (relative risk [RR] = 0.50, 95% CI= 0.27- 0.90, P< 0.05). Additionally, breastfed infants had fewer clinical manifestations, compared with infants who were not exclusively breastfed. The

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authors recommended exclusive breastfeeding as a means of preventing *Giardia* infections in highly endemic regions.

In studying the effects of breastfeeding on cryptosporidiosis among Cuban children with cryptosporidal diarrhoea, Pelayo *et al*. (2008) reported that 16 out of 25 (64%) had been breastfed as infants for a period ranging from 1- 6 months. There was a significant association between age-group and breastfeeding history. In this study, most of the older infected children (i.e. 5- 8 years) had been breastfed, whereas the majority of the younger children (aged 2- 5 years) had not. From analysis of their data, the authors suggested that maternal anti-*Cryptosporidium* antibodies acquired by an infant during breastfeeding may protect that child for the first few years of his or her life. As time elapses however, it appears that not only is that maternal protection lost but also the child that had been breastfed as an infant becomes even more susceptible to patent *Cryptosporidium* infection than a child of the same age who had only been bottle-fed. A further much larger study on this issue was recommended by the investigators of this study.

Two hundred and thirty-eight (238) Bedouin infants were followed from birth to age 18 months in a prospective cohort study (Bilenko *et al*., 2008). Observations made in this study shows that exclusive breastfeeding was protective against infection and morbidity by *Cryptosporidium* sp. and *G. lamblia* at ages 0 to 3 months. Also, in the age range of 4 to 6 months, partial versus non breastfeeding was associated with lower rates of infection with *Cryptosporidium* sp. (odds ratio, OR= 0.34, 95% confidence level CL, 0.18; 0.65). In older children (10-12 months) partial breastfeeding, as compared to none, protected against infections with *Cryptosporidium* sp. (OR = 0.57; CL, 0.36, 0.91) and *G. lamblia* (OR= 0.92, CI, 0.85; 0.99). The significance of their study was that, it encourages mothers to continue to

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at least partially breastfeed past age 3 months, as that may help reduce infections and morbidity in infants.

2.5.2.6 Socioeconomic factors and educational background of parents

Socioeconomic factors and the level of education of parents influence transmission of both *G. lamblia* and *Cryptosporidium* sp.. Wealth could be a marker for a family's ability to respond to new hardships. For instance, a less poor family may be better able to procure alternative sources of water if it becomes scarce and also afford basic necessities such as hygienic water, food, and quality healthcare. A relative index of socioeconomic status is usually constructed by combining household-level information on assets (Nundy *et al*., 2011). These include size and quality of housing and possessions. Information on the area of house, roof area, number of bedrooms per person, number of rooms per person, number of windows and number of doors, may all be relevant in assessing the socioeconomic status. Also, whether running water in house or outside house, flush toilet, and material for roofing are included. Possessions such as chairs, sofa, stools, closet, dresser, refrigerator, blender, gas stove, radio, TV set, and stereo sound system are included in the calculation of wealth index (WI).

Nundy *et al*. (2010) observed that there was a significant association between wealth and infection with *G. lamblia* among children studied during a longitudinal cohort study conducted in Peru. In the study, participants with greater wealth indices were associated with protection against *G. lamblia* ($P < 0.001$) and persistent *G. lamblia* infections (> 14 days). Their study provides useful information on how to control *G. lamblia* infections in impoverished settings in the face of limited resources. They suggested, this could be done by targeted interventions focused on poorer subpopulations. The assessment of relative wealth using household characteristics can be done quickly and with ease. In a similar study at

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Mexico however, Cedillo-Rivera *et al.* (2009) reported that, no association was found between seropositivity of *G. lamblia* infections and socioeconomic variables.

Mehraj *et al.* (2008) reported that socioeconomic status of a family was a risk factor for intestinal parasites including *G. lamblia* among children living in an urban slum of Karachi. In that study, rented house was taken as a proxy measure of socioeconomic status. The authors explained that the effect of socioeconomic status on risk of infections is complex involving several other factors such as lack of access to clean water, poor hygiene, lack of access to education due to financial constraints, and overcrowded conditions. They encouraged the government to enhance the activity of poverty reduction programmes.

To ensure that children are adequately cared for at home, personal hygiene and health education are critical. Unfortunately, it appears that parents who are not well educated either do not pay much attention to them or are completely ignorant of basic hygiene practices that could prevent infection of these parasites. A number of studies have shown close association of infections of both *G. lamblia* and *Cryptosporidium* sp. with the level of education of parents. Among Iraqi children suffering from acute diarrhoea, Hussein (2010) observed that the rate of *G. lamblia* was significantly associated with level of education of mothers as follows: illiterate or incomplete primary education (26.9%), complete primary or secondary education (22.3%), and high school or university education (9.7%). As indicated, infection was higher in children whose mothers had low level of education. The only risk factor identified with *G. lamblia* infection in a birth- cohort study conducted among Israeli Bedouin infants was maternal education (Fraser *et al*., 2000). The authors considered this observation as an indicator for maternal behaviour, and explained that hygienic conditions of food and

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utensil storage protect against *G. lamblia* infection, and these depend at least in part on maternal behaviour.

Multiple symptomatic *Cryptosporidium* infections was significantly associated with low socioeconomic status (P=1.000) and no formal education for mother (P= 1.000) among children in a semi-urban slum in India (Ajjampur *et al*., 2010).

2.5.2.7 Presence of domestic animals at home

With the recent application of genotyping tools in medical research, many studies have indicated that transmission of giardiasis and cryptosporidiosis could be zoonotic or anthroponotic, or both (Wang *et al*., 2011; Khan *et al*., 2010; Foronda *et al*., 2008). However, it is difficult to explain whether mere association or contact with domestic or companion animals or pets can lead to infection of any of these parasitic infections. Basically, the main mode of infection of *G. lamblia* and *Cryptosporidium* sp. is by consumption of water or food that is contaminated by cysts or oocysts of the parasites (Filice, 1952). Infected domestic animals could contaminate the immediate surroundings of human habitations with cysts or oocysts by defaecation in the environment. Therefore, in homes where there are dogs, cats, and other pets, a good personal hygiene and environmental sanitation could play a significant role in avoiding contamination of water or food with cysts and oocysts of *G. lamblia* and *Cryptosporidium* sp. respectively. Reports from past studies show contradictory observations on whether or not the presence of domestic animals has influence on transmission of giardiasis and cryptosporidiosis.

Studies carried out among Cuban children with diarrhoea (Pelayo *et al*., 2008) indicate that the most frequently reported animal contact was with dogs (64%), but contact with cats (18%), chickens (14%), pigeons (21%), pigs (7%) and cattle (4%) was also reported. No

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animal contact was recorded for 25% of the infected children. Upon genotyping 10 isolates of *Cryptosporidium* sp., all from children who had contact with animals at home, they were found to be *C. hominis* which is a human-specific genotype. The implication of this observation is that, the animal contacts listed (predominantly dogs) are unlikely to have been sources of *Cryptosporidium* infection.

In children hospitalized for diarrhoea in Goiania, Goias state in Brazil, Pereira *et al.* (2007) observed that giardiasis was positively associated with the number of cats per household, such that the odds for infection increased by about 25% for each additional cat in the household. Interestingly, the presence of one or more dogs in the household was not associated with the odds of giardiasis ($P > 0.10$). The odds of giardiasis was also about five and half times greater in diarrhoeic children who had lived on a farm within six months prior to visiting the hospital compared to diarrhoeic children who had not lived on the farm six months prior to the study. The authors explained that infected companion animals can excrete *G. lamblia* cysts in their faeces which could contaminate the surroundings in the home. The limitation of their study however, was that, the potential for zoonotic transmission from animals to human could not be measured, as has been done in some other studies through application of genotyping tools.

Contact with cats is one of the significant risk factors for *Cryptosporidium* infection in Indonesia (Katsumata *et al.*, 1998). The investigators in the study explained that this transmission could be through cat-human contact or environmental contamination by shedding oocysts. Khan *et al.* (2004) reported that there were no significant differences in *Cryptosporidium* infections between cases and controls ($P= 1.00$) with regards to contact with animals (cows, goats, chickens) in Bangladeshi children with diarrhoea. The occurrence of

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children from a daycare center in region of Presidente Prudente, Sao Paulo, Brazil presenting *G. lamblia* that had pets at home was low, and in none of the cases were pets suspected to be the source of contamination. This was because none of the pets presented *G. lamblia* cysts (Tashima *et al*., 2009). Pinheiro *et al.* (2011) however observed that pet ownership was significantly associated with the prevalence of giardiasis in the municipalites of Southeastern Minas Gerais State in Brazil. In a rural community in Thailand, Primary school children who had a history of contact with dogs more than once a week had a 2.3-fold greater risk for getting giardiasis infection (Ratanapo *et al*., 2008).

2.6 Laboratory diagnosis of *G. lamblia* and *Cryptosporidium* sp. infections

There are several methods used in the diagnosis of giardiasis and cryptosporidiosis (W.H.O., 1999; Tashima *et al*., 2009; Mark-Carew *et al*., 2010; Abe and Teramoto, 2011; Johnston *et al*., 2003), and these are categorized broadly into microscopy, immunological and molecular methods. Microscopy is considered to be the 'gold standard' for laboratory diagnosis of *G. lamblia* and *Cryptosporidium* sp., against which all other methods are evaluated because of its high specificity and sensitivity (Weitzel *et al*., 2006; Den Hartog *et al*., 2013). The procedure involves staining of slide preparations, which allows the morphological features of the two pathogens to be clearly seen on the slide (Mehraj *et al*., 2008; Mirzaei, 2007). Apart from using microscopy for screening, permanently stained prepared slides can be useful for demonstration and teaching purposes (W.H.O., 1999). Microscopy is used widely at many hospitals in developing countries (Adjei *et al*., 2003; 2004; Wellington *et al*., 2009; Nchito *et al*., 1998; Mukherjee *et al*., 2009) because its application is comparatively affordable. The other methods have rather been used in most cases for epidemiological studies or research purposes than routine diagnosis at the hospitals and clinics.

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Immunological methods, which depend on antigens of the parasites present in stool samples for detection of infection (Stibbs *et al*., 1998) are faster, and more sensitive (Al-Saeed and Issa, 2010), especially in cases where infection or parasite dosage is very low. A number of test kits whose principle of diagnosis is based on immunological principles have been produced commercially for use (Johnston *et al*., 2003; Weitzel *et al*., 2006). Some of these commercial kits upon evaluation however, have shown to be less sensitive to the conventional microscopy method (Weitzel *et al*., 2006). Currently, there are immunological assays which are capable of detecting multiple protozoan infections simultaneously from a single test (Den Hartog *et al*., 2013). The *TRI-COMBO PARASITE SCREEN* (TechLab, Inc. Blacksburg, VA) which was produced to detect three parasites namely *Giardia*, *Cryptosporidium* sp., and *E. histolytica* simultaneously was recently used in a study at rural paediatric clinic in Guatemala (Den Hartog *et al*., 2013). Comparing results obtained using this assay with that which was observed for microscopy in this study, the authors explained that the two methods agreed very well statistically, with a *Kappa* coefficient of 0.90.

The recent application of molecular tools in the detection of *Giardia* and *Cryptosporidium* sp. in stool samples (Asher *et al*., 2011; Nichols *et al*., 2003; Johnson *et al*., 1995) has not only improved upon diagnosis of these parasites but also helped to determine sources of infection through genotyping and sub-genotyping procedures (Insulander *et al*., 2013; Abe & Teramoto, 2011; Foronda *et al*.,2008). The polymerase chain reaction, PCR has been used extensively in the diagnosis of these enteropathogens. The method since its introduction into medical research, keeps improving as researchers continue to make modifications in the original protocol to achieve better results. The nested and seminested (or heminested) PCRs have been developed to improve upon the sensitivity and the general performance of the PCR technique. In these types of PCR, there are two separate reactions in the DNA amplification

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procedures, namely the primary and secondary reactions, in which the PCR product of the primary reaction is used as a template for the secondary reaction (Boontanom *et al*., 2010; Read *et al*., 2004; Sulaiman *et al*., 2003; Amar *et al*., 2002).

Another modification to the original PCR protocol is the Real-time PCR which is one of the modern molecular tools which combines PCR, chemistry with fluorescent probe detection of amplified product in the same reaction. In this modern technique, both PCR and amplified product detection are completed in an hour or less, which is considerably faster than conventional PCR. A number of real-time PCR assays have been developed for the detection of protozoan pathogens in stool (Velasquez *et al*., 2011; Molloy *et al*., 2010; Parr *et al*.,2007). In spite of the advantages of PCR, many laboratories in developing countries find it difficult to use it because of cost of installing molecular laboratory facility and reagents for running tests. The multiplex real-time PCR is capable of detecting many pathogens simultaneously. This type of real-time PCR was used by Haque *et al*. (2007) to determine presence of *E. histolytica*, *G. lamblia*, and *Cryptosporidium* sp. in stool samples simultaneously. The authors pointed out that, the use of the procedure will have limited scope for routine diagnosis in developing countries because of cost.

2.6.1 Comparative detection of *G. lamblia* and *Cryptosporidium* sp. by Microscopy, Immunoassays, and Polymerase Chain Reaction (PCR)

There is evidence that, based on the type of diagnostic tool used, the results obtained from screening stool samples for *G. lamblia* and *Cryptosporidium* sp. may differ significantly from one another (Roberts *et al*., 2011; Berrilli *et al*., 2006; Singh *et al*., 2009; Enriquez *et al*., 1997; Goldin *et al*., 1990) or may agree without significant difference (Den Hartog *et al*., 2013; Yakoob *et al*., 2010; Tumwine *et al*., 2003). To determine prevalence accurately

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therefore, it will be very useful if each sample is tested by more than one diagnostic method. The challenge of using more than one diagnostic method however, for screening samples in developing countries will be the cost of running some of the tests which may not be easily affordable. For research purposes, many studies conducted in the fields of molecular epidemiology of giardiasis and cryptosporidiosis in developing countries have combined microscopy, which is cheaper with PCR (Popruk *et al*., 2011; Ajjampur *et al*., 2009; Anthony *et al*., 2007; Gatei *et al*., 2006; Neira-Otero *et al*., 2005). In all of these studies, initial screening of all the stool samples were done using microscopy, and only the positive samples used for PCR. This study design reduces the cost involved in the research, as compared to where all samples are initially screened with PCR or ELISA method.

2.6.2 Genetic loci and DNA amplification

Amplification of DNA in PCR are done by targeting specific genetic markers (loci). A number of such genetic loci exist for both *G. lamblia* and *Cryptosporidium* sp.. These include the triosephosphate isomerase (TPI) and glutamate dehydrogenase (GDH) genes, and the small subunit ribosomal RNA (SSU rRNA) (Sulaiman *et al*., 2003; Abe *et al*., 2005; Wielinger and Thompson, 2007). Other amplification targets for *Giardia* include, β-giardin, EF-1α and GLORF-C4 (Caccio *et al*., 2005). Likewise, genotyping and subgenotyping of *Cryptosporidium sp.* has also been achieved through the amplification of the *Cryptosporidium* oocyst wall protein (COWP) gene, 18SrDNA, Hsp70, Actin, β-Tubulin, GP60, Microsatellites, Minisatellites, and Extrachromosomal double-stranded RNA, (McLauchlin *et al*., 2000, Caccio *et al*., 2005).

In determining the zoonotic potential of *G. lamblia*, Sulaiman *et al*. (2003) used a two-step nested PCR protocol to amplify triosephosphate isomerase (TPI) gene. According to the

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authors, TPI gene was chosen because of the high heterogeneity displayed by *Giardia* sp. at the TPI locus. They observed from their study that the TPI gene is a good phylogenetic marker for analysis of the molecular evolutionary and taxonomic relationship of *G. lamblia* parasites. Although many researchers have used either TPI, β-giardin or GDH in their studies to successfully genotype *G. lamblia* (Wang *et al*., 2011; Boontanom *et al*., 2010; Anthony *et al*., 2007; Read *et al*., 2004), or the 18SrDNA or COWP to genotype *Cryptosporidum* sp. (Insulander *et al*., 2013; Wang *et al*., 2011; Friesema *et al*., 2011; Neira-Otero *et al*., 2005), some authors (Abe and Teramato, 2011; Amer *et al*., 2010) are of the view that, the use of only one genetic marker produces information that has limited discriminatory power, in terms of genotyping. In view of this, the use of a multi-loci analysis has been suggested for genotyping these parasites.

Popruk *et al*. (2011) used two markers, namely, the small subunit ribosomal RNA (ssrRNA) and glutamate dehydrogenase (gdh) for screening and genotyping of *G. lamblia* asymptomatic infections among Thai orphans living in and around Bangkok. The authors explained that, the ssrRNA gene has high copy numbers arranged in tandem repeats as well as a more conserved sequence, for which reason it is commonly used for screening. The gdh gene was used on the basis of being able to differentiate genetic assemblages using NlaIV and RsaI endonuclease enzymes in PCR-RFLP analysis.

2.6.3 Polymerase chain reaction- (Restriction Fragment Length Polymorphism) (PCR-RFLP)

The 'restriction fragment length polymorphism'(RFLP) analysis has been used together with the PCR technique to successfully genotype and subgenotype *G. lamblia* and *Cryptosporidium* sp. (Wang *et al*., 2010; Ajjampur *et al*., 2010; Singh *et al*., 2009; Parr *et al*.,

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2007; Gatei *et al.*, 2006; Houpt *et al.*, 2005). From several past studies, it has strongly and convincingly been indicated that the PCR-RFLP is a very useful genotyping tool, as in most cases results obtained are comparable with sequence analysis to a very large and acceptable extent (Read *et al.*, 2004; Sulaiman *et al.*, 2003; Amar *et al.*, 2002).

Through the application of the PCR-RFLP genotyping tool, Abe and Teramoto (2011) identified the first possible person-to-person transmission, when they observed that assemblage (or genotype) B was the only genotype detected in their study. The study was carried out among patients and health care workers at a rehabilitation institution for developmentally disabled people in Osaka city, Japan. The genotype B exclusively belongs to human infections which indicate that transmission of the infection was anthroponotic. The tool in this case enabled the researchers to trace source of infection.

In Tunisia, Essid *et al.* (2008) applied a nested PCR-RFLP analysis on *Cryptosporidium* isolates to identify the particular species involved in transmission of infections among children hospitalized for immunodeficiency. The authors observed that the isolates could be in three categories, namely *Cryptosporidium hominis*, *C. parvum*, and *C. meleagridis*, with *C. hominis* and *C. parvum* being higher in diarrhoeal specimen than in formed stools. Also, *C. hominis* was more prevalent in children from urban areas than those from rural areas. This demonstrates the usefulness of the PCR-RFLP molecular tool in providing an extensive knowledge on the epidemiology of cryptosporidiosis and giardiasis among a population. Similarly, application of PCR-RFLP genotyping tool in a study at the Philippines slum area in Manilla revealed that assemblages A and B were both present in the population, with assemblages B (86.47%) being more predominant over assemblage A (Anthony *et al.*, 2007). The researchers emphasized that knowledge of genotypes that were in circulation among this

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population provides clues in instituting control measures. They also pointed out that the results had expanded knowledge on epidemiological studies in the area.

The PCR-RFLP genotyping tool enabled Chappell *et al.* (2011) to detect *C. meleagridis* infection among human. The authors considered this observation as an unusual occurrence, because this particular species has been identified in less than 1% of persons with diarrhoea. *Cryptosporidium parvum* and *C. hominis* are the two main species that are often associated with human infections.

In order to determine the possibility of zoonotic transmission of *Cryptosporidium* sp. among Indian children living in Vellore, Southern India (Rajendran *et al*., 2011), diarrhoeal stool samples from the children, and some animals in the area were tested and analysed by the PCR-RFLP genotyping tool. The species in humans were identified as *C. hominis*, and subgenotyped as Ia, Ib, Ie, and Id). *Cryptosporidium andersoni* was also identified as the predominant species in livestock. In their study, none of the species identified in the children belonged to any animal group, indicating that none of the infections came from animals. However, *C. hominis,* which is normally seen in human was identified in the animal samples, which shows a possibility for cross species transmission.

At Mulago hospital, Kampala, Uganda, cryptosporidial diarrhoea was studied among diarrhoeic children over a 15-month period (Tumwine *et al*., 2003). In this study, the PCR-RFLP tool was utilized to determine the species involved in transmission in the area. The authors reported that *C. parvum* was the predominant species responsible for human cryptosporidial diarrhoea in the city of Kampala, Uganda. The extent of genetic diversity and transmission pathways of both *Giardia* and *Cryptosporidium* infections in the Kaduna state of

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Nigeria were studied by means of PCR- RFLP tool (Mikai *et al.*, 2012). Reports from that study indicate that *C. hominis* (Ia), *C. parvum* (IIe), *G. lamblia* (A2- subtype) were the commonest in the population. The authors recognized that the mode of transmission in this population was likely to be anthroponotic, as revealed by the genotyping results of their study. In an earlier study conducted at Osun State, Nigeria, similar techniques employed by Molloy *et al.* (2010) helped to determine the species, genotypes, and subgenotypes of *Cryptosporidium* sp. in the area. The investigators observed a high diversity of the parasite in the area, which include three species, namely *C. hominis*, *C. parvum*, and *C. meleagridis,* as well as *Cryptosporidium* rabbit genotype, the cervine genotype and *C. canis.* Most importantly, observations from this study showed that the source of infection could be anthroponotic, zoonotic and/ or environmental.

2.7 Clinical manifestations of *G. lamblia* and *Cryptosporidium* sp. infections

Many conflicting observations on clinical manifestations associated with *G. lamblia* and *Cryptosporidium* sp. infections have been reported (Yakoob *et al.*, 2010; Lindo *et al.*, 1998). Variations in these reports however could possibly be as a result of differences in immune status of individuals (Mahmud *et al.*, 2001), as well as parasite genotypes involved in the infection (Gelanew *et al.*, 2007; Insulander *et al.*, 2013).

In a study involving the etiology of diarrhoea in children less than 5 years who had been admitted at a rural hospital of Southern Mozambique (Mandomando *et al.*, 2007), vomiting was identified to be more frequent in children with *G. lamblia* infection. Among Cuban children with cryptosporidial diarrhoea, the most common symptoms apart from diarrhoea were loss of appetite (anorexia) in 68% of the children, abdominal pain in 57%, and nausea

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54%. Only 18% of the children were reported to have lost weight, and vomiting was only reported for 7% of the children (Pelayo *et al*., 2008). The authors of this study also observed that, the co-infection of *Cryptosporidium* sp. with *Giardia* did not influence either the number or type of symptoms recorded. A probable modulation of clinical symptoms by *Giardia* during coinfections with other enteropathogens has been suggested following observations of a study among Bedouin children (Bilenko *et al*., 2004).

A 4- year longitudinal birth cohort study of cryptosporidiosis among children in Peru (Cama *et al*., 2008) indicates that 36% of infected children had diarrhoea, 28.4% had general malaise, 16.5% had abdominal pain, 15.7% had vomiting, and 7.9% had nausea. None of the children in the study reported fever or blood in stools. Additionally, pattern of clinical manifestations was observed to vary among *C. hominis* subtype families. In the study, infections with subtype family Ib were associated with nausea, vomiting, general malaise, and diarrhoea whilst infections with other subtype families (Ia, Id, and Ie) were generally associated with diarrhoea only. In another study in Jamaica (Lindo *et al*.,1998), apart from diarrhoea, there were no other identifiable clinical manifestations associated with *Cryptosporidium* infection.

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2.8 Genetic variability of *G. lamblia* and *Cryptosporidium* sp., and association with diarrhoea

In countries where modern laboratory facilities exist for molecular studies, researchers have made use of genotyping tools in epidemiological studies to investigate whether there is any association of identified genotypes of *G. lamblia* and *Cryptosporidium* sp. with diarrhoea and other symptoms. For instance, *G. lamblia* assemblage (genotype) B was found to be associated with flatulence among Swedish children (Lebbad *et al*., 2011). So far, information available indicate that some level of contradiction on the subject of whether there is an association of specific genotypes with diarrhoea exists.

Giardia assemblage (AI) was found to be the predominant genotype for diarrhoea among Peruvian children (Perez Codon *et al*., 2008). Although assemblage B was also identified in their study, it was not associated with diarrhoea. On the contrary, Al-Mohammed (2011) reported recently that whilst all assemblage B cases were associated with symptomatic giardiasis among Saudi children, assemblages AI and AII were asymptomatic. Similarly, all diarrhoeal cases identified in a study at Osaka city were caused by *Giardia* genotype B, with none at all from genotype A (Abe and Teramato, 2011). In Egypt, although both genotypes A and B were identified to cause giardial diarrhoea, there were more infections of genotype B (80%), than genotype A (15%) (Foronda *et al*., 2008). Similar observations were made among Indian children (Ajjampur *et al.,* 2009) and Ethiopian children (Gelanew *et al*., 2007), all showing predominance of *Giardia* assemblage B in diarrhoeal cases.

With regards to cryptosporidial diarrhoea, Pelayo *et al.* (2008) observed that all the infections identified in their study were *C. hominis*, belonging to subtypes Ia, Ib, and Id. In another

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study among Peruvian children in which cryptosporidial diarrhoea was studied (Cama *et al.*, 2008) it reported that, all three species, *C. hominis*, *C. parvum*, and *C. meleagridis* could cause diarrhoea, as well as subtypes Ia, Ib, Id, and Ie. The authors remarked that the role of parasite genetics with regards to clinical manifestations was not clear. According to them, variations in clinical manifestations in their study occurred according to the subtypes, and that, Ib subtype appeared to be most virulent. In Nigeria, Molloy *et al.* (2010) observed that *C. hominis* was the most prevalent species, followed by *C. parvum.* Other species such as *C. meleagridis* were also seen in smaller proportions. In their reports however, nothing was stated about the association of genotypes with clinical manifestations.

2.9 Co-infections and Clinical manifestations

In sub-Saharan Africa and many developing countries large populations are usually exposed to several risk factors, which could lead to multiple infections, also referred to as co-infections. Co-infection of enteropathogens have been identified in several epidemiological studies, both in communities and hospitalized cases (Mukherjee, 2009; Pinheiro, 2011; Lebbad *et al.*, 2011). The clinical manifestations associated with co-infections are varying, which include mild or no symptoms (Friesema et al., 2011) and severe forms (Fincham *et al.*, 2002; MacDonald *et al.*, 2002).

Various researchers in epidemiological studies have suggested a possibility of influence that one enteropathogen could have on another if they co-exist during a multiple infection of an individual, which could be biochemical or immunological. For instance, the subject of immuno-modulation associated with helminthic co-infection with other enteropathogens in patients has been investigated at many places including Bangladesh (Harris *et al.*, 2009).

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Although there were no significant differences in the vibriocidal or lipopolysaccharide (LPS)-specific immune responses to *V. cholera*, helminth-infected cholera patients had decreased faecal and serum IgA immune responses to the B subunit of cholera toxin (CTB) as well as a more modest in serum IgG response to CTB.

A typical case of co-infections which has attracted much attention is co-infections of enteropathogens with *G. lamblia*. In a mouse model experiment, it was observed that *G. lamblia* could bind significant amounts of cholera toxin reducing the toxin effect (Ljungstrom *et al*., 1985). Based on this report, Bilenko *et al.* (2004) investigated the ability of *G. lamblia* to modulate the effect of other pathogens in co-infections to modify the clinical expression of disease among Bedouin infants. They reported that, there were no differences in severity between episodes in which enteric pathogens were detected alone, compared to episodes in which the same pathogen was found together with *G. lamblia*. They observed however that there was an exception in the case of rotavirus infections, and that, episodes with rotavirus alone were more severe as compared to episodes when rotavirus was co-infected by *G. lamblia*. From this study, the authors recommended further investigations into the modulation of clinical symptoms resulting from *G. lamblia* co-infection.

Rotavirus infection is now one of the leading causes of gastroenteritis in children worldwide including Ghana (Cunliffe *et al*., 2002; Armah *et al*., 2005; 2006; Reither *et al*., 2007), and children of age 2 years and below are most affected (Widdowson *et al*., 2000; Nerurkar, 2011). Rotavirus gastroenteritis is associated with high morbidity in developed countries and significant mortality in developing countries (Saravanan *et al*., 2004; Ramani and Kang, 2007; Chang *et al*., 2003).

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In Puerto Rico, laboratory diagnosis of rotavirus has recently been given a serious attention so that rates of infection can accurately be reported (Roman and Martinez, 2005). Observations made so far, are that, co-infection of *G. lamblia* and rotavirus is common in the Puerto Rican population. The stool consistency of the *G. lamblia* and rotavirus positive samples in their study was reported to be more soft than watery, similar to the consistency observed in most of the rotavirus only positive samples. This implies that having an infection with two enteropathogens apparently did not worsen the clinical symptoms. The clinical symptoms of *G. lamblia* co-infections with other enteric pathogens including rotavirus and *Cryptosporidium* have also been studied in an urban slum in south India (Ajjampur *et al*., 2009). Reports showed that children with co-infections have a slightly longer duration of diarrhoea.

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CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area and Population

The study was conducted mainly at the Princess Marie Louise Children's hospital (PML) at Accra, Ghana and partly in a few daycare centers also located in Accra. The hospital is the main paediatric health facility located within the metropolitan area of the city and accessed by people from all parts of Accra. These include people from different socio-cultural, economic, political or educational background.

The hospital has both out-patients and in-patients departments which are fully and well patronized by patients. According to the hospital's recent report, the daily average attendance of patients to the hospital is 143. This comprises of an average of 85 new attendants, and 58 old attendants. It is estimated that about 90 percent of these patients are children below 5 years, of which about 11 percent report with diarrhoea and other related problems.

The set-up of the hospital includes a recovery ward, an outpatient department where patients are sorted out for attention, four in-patient wards with a 25 bed capacity each, emergency detained wards (30 beds), a dispensary and a laboratory as well as a records and administrative unit, an injection room, a catering unit, public health unit, a recreational area for children, and a mortuary. At the time of undertaking this study, the staff of interest population was 6 doctors (2 specialist, 4 senior medical officers (SMO), and 1 medical officer on contract, 5 medical assistants (1 principal medical assistant, 1 senior medical assistant, 2 grade one medical assistant, 1 grade two medical assistant), 2 pharmacists with attendants, and 53 nurses and 13 reproductive and child health officers.

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The study population for the hospital-based study (patients) were children (of age 5 years and below) who had been hospitalized at PML primarily due to acute diarrhoea and dehydration. Diarrhoea was defined as passage of loose or watery stools in the previous 24 hours and still present when the faecal specimen was collected at the hospital. Patients who developed diarrhoea after admission at the hospital, as well as patients with diarrhoea resulting from food intolerance were all excluded from the study. Some children who were hospitalized at the same hospital within the period of study for medical conditions other than diarrhoea were included as control subjects.

The community-based study was also carried out among apparently healthy children of age 5 years and below, in randomly selected daycare centres in the Korle Gonno community, Accra. The Korle Gonno community which has a population of over 100,000 inhabitants (http://ama.gov.gh/ama/page/5052/sub-metro), is located about 3 km from the Children's hospital. Korle Gonno is a densely populated community with a lot of sanitary challenges including chocked gutters and a dirty environment especially during the rainy season. The main source of drinking water in the community is mostly pipe borne and sachet water. Most homes lack toilet facilities, leading to indiscriminate urinating and defaecating in the open. Also, many homes have domestic animals including dogs and cats.

In all, 485 patients were referred to the study by the paediatricians and nurses at the wards, of which 365 were patients with diarrhoea, and 120 being patients without diarrhoea, that were used as controls. A total of 119 stool samples were also collected during the community-based study from children in the day care centres.

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3.2 Study design

It was both hospital and community-based prospective cross-sectional study, which was conducted between the periods of March, 2010 and June, 2011. The design of the study allowed for the detection and comparison of types of genotypes (or assemblages) occurring in both symptomatic and asymptomatic infections among children in Accra. One of the objectives of the study was to investigate whether there is any association between type of genotype and disease manifestation.

An ethical approval of the study was obtained from the Research and Ethical Review Committee of the University of Ghana School of Allied Health Sciences, College of Health Sciences, Korle bu, Accra. The inclusion of participants (patients and healthy children) in the study was strictly voluntary and based on informed consent of their guardians or parents, after being informed of the objectives and goals of the study.

To investigate whether or not there is a modulating effect of clinical symptoms of rotaviral diarrhoea during co-infection of rotavirus and *G. lamblia,* separate samples collected and stored frozen from an on-going rotavirus project were used. At the end of the study, research information gathered included laboratory findings (microscopy, immuno- assays, molecular assays (genotyping), clinical and demographic data for both diarrhoeal and non-diarrhoeal cases.

3.3 Demographic and clinical information

Demographic and clinical data were obtained by a study nurse at the hospital ward by means of a structured questionnaire (a copy in appendix 1). In the community daycare centres, nursery attendants and teaching assistants assisted in collecting demographic data.

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Demographic data included location of individuals within the city (i.e. residence), sex, age, parents occupation, parents educational background, source of drinking water, breastfeeding habits, whether food is prepared at home or obtained from the street, and presence of domestic animals at home. Clinical data included vomiting, malaise, fever, abdominal pain, consistency of stool, and frequency of passage of stool, drugs used for treatment or management, as well as number of days of hospitalization (for children hospitalized only).

3.4 Sample collection and detection of *G. lamblia, Cryptosporidium* sp. and rotavirus

A single stool sample was collected from each patient by parents or guardians and placed into a clean disposable plastic tube with tight fittings. The consistency of the stool was directly observed and classified and recorded by the study nurse as either loose, semi-formed, formed, mucoid, slimy, or watery. Each sample was then divided into two portions, with one portion preserved in 10% formalin whilst the other without the addition of any reagent or preservative was stored frozen at a temperature of – 20°C. In this study, each sample was tested by laboratory methods, namely, microscopy and immunoassay tests for *G. lamblia* and *Cryptosporidium* sp., after which all positive samples together with some randomly selected negative samples were tested by the polymerase chain reaction (PCR). Rotavirus screening was done by the use of immunoassay kits only.

3.4.1 Stool microscopy

Each stool sample preserved in 10% formalin was processed by direct smear slide preparations and formol-ether concentration procedures for *G. lamblia* cysts, as well as the modified Ziehl-Neelsen technique for *Cryptosporidium* oocysts, for observation under the

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microscope (WHO Parasitology Laboratory manual, 1991). Other intestinal parasites were also looked out for in these samples.

3.4.1.1 *Giardia lamblia* cysts and trophozoites detection

A drop of diarrhoeal stool sample (approximately a match-head size of formed stool sample) was separately added to iodine and saline drops placed at the two ends of a microscope slide, and mixed thoroughly with an applicator stick. They were covered with cover slips and examined under a microscope with X10 objective for either trophozoites or cysts of *G. lamblia*.

To perform the formol-ether concentration technique, 10 ml of 10% formalin was added to approximately 1 gm of stool sample and stirred using an applicator stick until a slightly cloudy suspension was obtained. The stool suspension was filtered through a gauze layer placed in a funnel, into a centrifuge tube until the 7 ml mark was reached. The filter was removed and discarded, and 3 ml of ether or ethyl acetate added and mixed well. The resulting mixture was centrifuged at 1000xg for 10 minutes, and the supernatant poured away. The sediment was well mixed and a drop transferred onto a slide for examination under the microscope. Each preparation was stained with iodine before observation under an optical microscope for the cysts.

3.4.1.2 *Cryptosporidium* sp. oocyst detection

The modified Ziehl-Neelsen method was used to detect *Cryptosporidium* sp microscopically. A faecal smear was made on a slide, and left to air-dry, after which it was then fixed in methanol for 2-3 minutes. The smear was stained with cold carbol-fuchsin for 5- 10 minutes, and the preparation was then differentiated in 1% hydrochloric acid – ethanol until colour

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ceased to flood out. It was rinsed in tap water, drained dry, and examined, first with high-power objective of an optical microscope, and the morphology confirmed using oil immersion. The oocysts appeared as bright rose-pink spherules against a pale green background. All the samples which tested positive for *Cryptosporidium* sp. were selected for further analysis by PCR.

3.4.2 Enzyme Immunoassay tests

The enzyme immunoassay tests were performed on cold faecal samples (samples stored at a temperature of -20°C), by use of the Wampole™ *GIARDIA/CRYPTOSPORIDIUM* CHECK® ELISA KIT (TechLab Inc. Blacksburg VA) for the two parasites, and ProSpecT™ Rotavirus Microplate Assay (Oxoid Ltd. Wade Road Basingstoke Hants, UK) for rotavirus infection. The test involves a qualitative detection of *Cryptosporidium*/ *Giardia* or rotavirus using monoclonal antibodies to the *Cryptosporidium* oocyst, *Giardia* cyst or rotavirus antigens in human faecal samples. There are separate test kits specifically for *Giardia*, *Cryptosporidium* and rotavirus antigen detection, which however operate on the same principle.

3.4.2.1 Description of enzyme immunoassay kits and principle of test

The components of the kits are as follows:

(i) Conjugate (rabbit polyclonal antibody to a *Cryptosporidium* oocyst /*Giardia* cyst/ rotavirus antigen coupled to horseradish peroxidase in a protein buffered solution containing 0.02% thimerosal).

(ii) Diluent (buffered protein solution containing 0.02% thimerosal),

(iii) Stop solution (0.6N sulphuric acid),

(iv) Positive control (heat-inactivated bovine faecal material containing *Cryptosporidium* oocyst/*Giardia* / rotavirus antigen in a protein buffered solution with 0.02% thimerosal).

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(v) Negative control (Tris buffered saline solution)

(vi) Substrate (solution containing tetramethylbenzidine and peroxide),

(vii) Wash buffer concentrate (phosphate buffered saline, detergent and 0.2% thimerosal), and (viii) Microassay plate (12 strips, each consisting of 8 wells coated with monoclonal antibody.

The microassay plate in the kit contains an immobilized monoclonal antibody against *Cryptosporidium* oocyst or *Giardia* cyst or rotavirus antigen. In the assay, an aliquot of a diluted faecal sample is transferred to a microassay well. If *Cryptosporidium* oocyst or *Giardia* cyst or rotavirus antigen is present, it binds to the immobilized monoclonal antibody. Upon addition, the conjugate then binds to the antigen/antibody complex. Any unbound materials are removed during the washing steps. Following the addition of a substrate, a colour is detected due to the enzyme –antibody-antigen complexes that form in the presence of *Cryptosporidium* oocyst or *Giardia* cyst or rotavirus antigen.

3.4.2.2 Procedure of test

The Enzyme immunoassay test was performed by following a number of steps, which were the same for *Cryptosporidium*, *Giardia* and rotavirus detection in the manufacturer's instructions. The frozen samples were thawed. For each sample, a 400 µL volume of Diluent was added to a microcentrifuge tube, and 100 µL of the diarrhoeal stool sample (or approximately 0.1 gm of formed or semiformed sample, about the size of a small pea) added. The resulting solution was then well mixed.

To run the test, two wells were selected on the microassay plate, to be used as controls, one as positive control and the other as negative control. The rest of the wells were considered as test wells. A 100 µL volume of diluent was transferred into each test well, after which 50 µL

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of prepared stool sample was added and gently tapped to mix. After filling all the test wells with test samples, 50 µL of positive control and 100 µL of negative control were added to their respective wells. The plate was well sealed with a plate sealer and incubated for 1 hour at room temperature. The contents of the wells were discarded after the period of incubation, the wells were washed thoroughly, and slapped on a dry towel to completely remove any traces of wash solution. The washing process was carried out four times. In the next step, 50 µL of the Conjugate was added to each well and gently tapped to mix and then sealed with a plastic adhesive sheet. It was incubated for 30 minutes at room temperature, after which the contents of the plate was discarded and washed as described previously. After washing and thorough removal of all traces of solution, 100 µL of Substrate was added to each well, and gently tapped to mix. There was a final incubation of wells at room temperature for 10 minutes. In the final step, 50 µL of Stop solution was added to each well and gently tapped to mix contents. The stop solution changes the blue colour formed to a yellow colour. Results were read both visually by assessing the colour formed in each well, and quantified by measuring the absorbance at 450 nm on a microplate ELISA reader (Labsystems Multiskan MS, Finland, serial RS- 232C).

3.4.2.3 Interpretation of results

Visual

Any sample that was colourless or resembles the negative control well in intensity of colour (i.e. clear to slight yellow) was interpreted as negative. It implies that if the well contained any antigen at all it was below the detectable limits of the assay.

On the other hand, any sample that was obviously more yellow than the negative control well (i.e. pale yellow to strong yellow) was positive.

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Spectrophotometric

From a print-out of the test results, obtained from the ELISA reader (Labsystems Multiskan MS, Finland, serial RS- 232C), the absorbance values of both the negative and positive controls were determined. The recommended values (cut-off points) were <0.150 OD_{450}, for the negative control, and ≥0.500 for the positive control. In the test wells, any value greater than, or equal to 0.150 (≥ 0.150) was considered positive, and recorded.

3.4.3 Polymerase chain reaction (PCR)

All samples which tested positive for *G. lamblia* or *Cryptosporidium* sp., or both during microscopy and immunoassay test were selected for the PCR test. Additionally, some samples which tested negative for both pathogens were randomly selected and included in the PCR test. The reason for performing the PCR test was to compare its detection rate with the other methods already used in this study, and also to obtain a PCR product from which genotyping reactions could be performed.

3.4.3.1 DNA extraction and purification

Giardia or *Cryptosporidium* DNA was directly extracted from the stool samples by using the MO BIO UltraClean®Fecal DNA Isolation kit. The procedure was performed by following the manufacturer's instructions. The steps involved were meant to completely break or dissolve the wall of the (oo)cysts to release the DNA into solution. Briefly, about 250 mg (or 250 µl of liquid) stool sample was added to a 2 ml Dry Bead Tube, after which 550 µl of Bead Solution was added. This was vortexed gently to mix. There was further addition of 60 µl of pre-warmed Solution S1, and after mixing, 200 µl of Solution IRS (Inhibitor Removal Solution) was added. The resulting mixture was vortexed at a maximum speed for 10 minutes, and also centrifuged at 10,000g for 30 seconds. The supernatant was transferred to a

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clean 2 ml Collection Tube, and 250 µl of Solution S2 added, vortexed for 5 seconds and incubated at 4°C for 5 minutes. After incubation, the resulting mixture was centrifuged at 10,000g for 1 minute, and 450 µl of the supernatant transferred into a 2 ml Collection Tube. This was followed by the addition of 900 µl of Solution S3 to the supernatant, vortexed for 5 seconds, after which 650 µl of the mixture was transferred onto a Spin Filter and centrifuged at 10,000g for 1 minute. Next, 300 µl of Solution S4 was added and centrifuged again at 10,000g for 30 seconds. The flow through was discarded and the tube centrifuged again for 1 minute. In a final step, the Spin Filter was carefully placed in a new 2 ml Collection Tube and 50 µl of Solution S5 added to the centre of the white filter membrane, centrifuged at maximum speed for 30 seconds after which DNA was obtained. The filter was discarded, and the DNA stored frozen (-20°C to -80°C) until it was used in PCR.

3.4.3.2 Polymerase chain reaction amplification and detection for *G. lamblia*

A nested PCR amplification of *G. lamblia* gdh gene using specific primers was performed in an Applied Biosystem thermocycler (USA), model 2720, following previously described methods by Boontanom *et al.* (2010), which was a little modification to Read *et al.* (2004). To amplify *gdh*, a primary external forward ripmer, GDH1a (5'ATCTTCGAGAAGGATGCT TGAG3'), GDH1 (5'ATC TTC GAG AGG ATG CTT GAG3') and external reverse primer, GDH5s (5'GGATACTTSTCCTTG AACTC3') were used to produce a 2324 bp fragment. In the secondary PCR, a 461 bp of *gdh* was amplified using GDHeF (5'TACACGTYAAYCGYGGYTTCCGT3') and GDHiR (5'GTTRTCCTTGCACATCTCC3').

The first-round PCR amplification was performed using mixtures of 1U of *Taq* polymerase with 1X PCR buffer, 1.5 mM $MgCl_2$, 200 µM each of dNTP, 500 nM of each primer, and 3

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µl of the extracted DNA in a total volume of 25 µl. The thermal cycling conditions were performed for 35 cycles as follows: 94ºC for 7 minutes, and then 94ºC for 1 minute, 55ºC for 1 minute, and 72ºC for 1 minute, with a final cycle of 72ºC for 7 minutes.

The second-round PCR was performed using mixtures of 1U of *Taq* polymerase with 1X PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 500 nM of each primer, and 1 µl of the primary PCR product in a total volume of 25 µl. The thermal cycling was initiated with 1 cycle of 94ºC for 2 minutes, 56ºC for 1 minute, and 72ºC for 2 minutes, followed by 55 cycles of 94ºC for 30 seconds, 56ºC for 20 seconds, 72ºC for 45 seconds, and final extension at 72ºC for 7 minutes. The PCR products were analyzed by 2% agarose gel-electrophoresis, stained with ethidium bromide and then visualized on a UV transilluminator.

3.4.3.3 Polymerase chain reaction amplification and detection for *Cryptosporidium* sp.

Cryptosporidium sp was detected from the frozen faecal samples by following methods previously described by Nichols *et al*. (2003) with slight modification.

The protocol which was a nested PCR, involved amplification of the *Cryptosporidium* sp *18S rRNA* gene using specific primers. In the primary reaction, an external forward primer, outN-DIAGF2(CAATTGGAGGGCAAGTCTGGTGCCAGC) and external reverse primer, outN-DIAGR2 (CCTTCCTATGTCTGGACCTGGTGAGT) were used to produce a 655 to 667 bp fragment. In the secondary PCR, a 435-bp of *18S rRNA* was amplified using inDIAGF (AAGCTCGTAGTTGGATTTCTG) and inDIAGR(TAAGGTGCTGAAGGAGTAAGG).

The first-round PCR amplification was performed in an Applied Biosystem thermocycler (USA), model 2720, using mixtures of 5U of *Taq* polymerase with 1X PCR buffer, 50 mM $MgCl_2$, 10 µM each of dNTP, 10nM of each primer, and 5 µl of the extracted DNA) in a

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total volume of 25 µl. The thermal cycling conditions were performed for 30 cycles as follows: 95ºC for 5 minutes, and then 94ºC for 30 seconds, 58ºC for 1 minute, and 72ºC for 30 seconds, with a final cycle of 72ºC for 10 minutes. Samples were then cooled to 4°C.

The second-round PCR was performed using mixtures of 5U of *Taq* polymerase with 1X PCR buffer, 50 mM $MgCl_2$, 10µM of each dNTP, 10 nM of each primer, and 1 µl of the primary PCR product in a total volume of 25 µl. The thermal cycling conditions were performed for 30 cycles as follows: 95ºC for 5 minutes, 94°C for 30 seconds, 58ºC for 1 minute, and 50ºC for 1 minute, and final extension at 72ºC for 10 minutes. The PCR products were analyzed by 2% agarose gel-electrophoresis, stained with ethidium bromide and then visualized on a UV transilluminator.

3.4.3.3.1 Genotyping of *G.lamblia* isolates by PCR-RFLP analysis

G. lamblia genotypes were determined by PCR-RFLP in the region of the glutamate dehydrogenase (gdh) gene, as described by Read *et al.* (2004), using *Nla*IV. This technique was selected on the basis of being able to differentiate subgroups (AI, AII, and B) within the genetic assemblages. Restriction digests were carried out directly on PCR products in 20 µl reactions. Ten microliters of PCR product were added to 1X reaction buffer, 0.2 µg/µl BSA and 4 U NlaIV (Biolabs Inc., New England), and digestion took place at 37°C for 3 hrs. The PCR products and a ladder of 100 bp (Sigma, USA) were electrophoresed in a 2.0% agarose gel and visualized by ethidium bromide staining.

3.4.3.3.2 Genotyping of *Cryptosporidium* sp. isolates by PCR-RFLP analysis

Ten microliters of the PCR product was digested for 30 minutes at 37°C with 10 U of each restriction enzyme, VspI and SSpI (Thermoscientific, EU, Lithuania) in 35 µl of 1X buffer.

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Digested products were electrophoresed in a 2.0% agarose gel and visualized by ethidium bromide staining.

3.4.3.3.3 Sequencing

Purification and sequencing of the PCR products were done commercially (at Macrogen Europe Laboratory IWO, Kamer 1A3-195, Meibergdreef 39, 1105, AZ, Amsterdam, Netherlands). The accuracy of data was con rmed by two-directional sequencing with the forward and reverse primers used in secondary PCRs. Chromatograms and sequences were analysed using Chromas (http://www.technelysium.com.au/chromas. html). Sequences were compared to reference sequences downloaded from the GenBank using ClustalW.

Sequencing allowed assessment of the extent of genetic diversity within *G. lamblia* , and also in *Cryptosporidium* sp. parasites. It was also meant to confirm the genotyping results obtained from PCR-RFLP analysis.

3.5 Clinical data for rotavirus-giardia co-infection study

Clinical data collected along stool samples to assess whether *Giardia* is able to modulate clinical symptoms of rotaviral diarrhoea during co-infection of the two pathogens was more detailed and included the following:

a. description of child's illness by parent/guardian (diarrhoea, vomiting, fever)
b. Duration of symptoms before admission
c. Severity of symptoms during admission (how many stools during the day/ how many stools during the night, when diarrhoea was at its worst).

Also, how many vomits during the day/ how many vomits during the night, when vomiting was at its worst.

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d. Severity of symptoms by Physicians clinical assessment on admission (sunken eyes, lethargic, sunken anterior frontanelle, mild dehydration, moderate dehydration, severe dehydration)
e. Management and duration of hospitalization.

Severity score for all diarrhoeal episodes were computed according to procedures previously described by Bilenko *et al*. (2004), and results matched with laboratory data.

3.6 Statistical analysis

All data were analysed using Version 17.0 of the SPSS Software Package (SPSS Inc. Chicago, IL) and *Stata*/SE 11.1 Statistical Software (Stata Corporation, Texas, USA). The results are expressed as means (S.D.) for the continuous variables (such as age) and as numbers and percentages of the subjects, or two groups of subjects, for the categorical data (such as gender, positivity in one of the investigations, presence of diarrhoea, etc). Univariate analysis was performed using independent-sample t-test, Pearson X^2 test or Fisher's exact tests, as appropriate. A P-value of < 0.05 was considered statistically significant difference or association.

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CHAPTER FOUR

RESULTS

4.1 Epidemiology of *G. lamblia* and *Cryptosporidium* sp.

The epidemiology of *G. lamblia* and *Cryptosporidium* sp. were studied at the Princess Marie Louise children's hospital (PML), Accra, and also within selected daycare centres located at the Korle Gonno community, Accra. The results obtained from the study indicate that both parasites were present among a number of children at the hospital and also in the daycare centres.

4.1.1 Hospital-based study

A total of 485 stool samples, comprising of 365 diarrhoeal cases (symptomatic), and 120 non-diarrhoeal cases were collected from patients who were hospitalized at Princess Marie Louise children's hospital (PML), Accra. All the samples were screened for *G. lamblia* and *Cryptosporidium sp.* as well as other enteric parasites by microscopy, and by enzyme immunoassay kits to determine *G. lamblia, Entamoeba histolytica* and *Cryptosporidium sp* only. The results of stool testing for the presence of these parasites are described in Fig. 4.1. The infection was highest for *Cryptosporidium* sp., followed by *G. lamblia*, and then a low infection rate for *E. histolytica*.

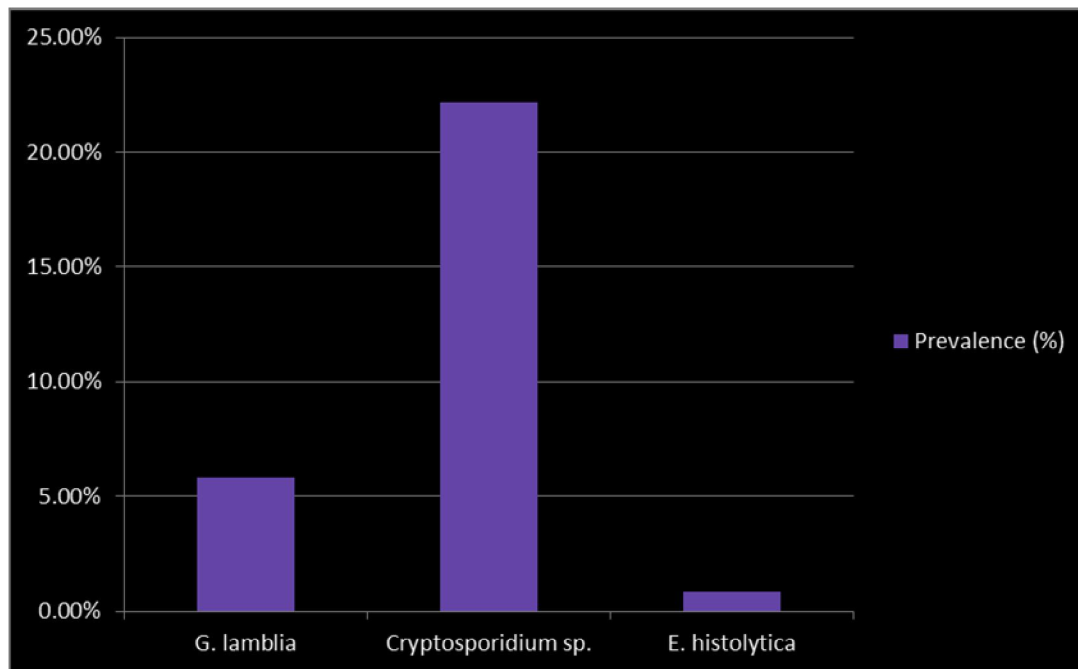

Figure 4.1 Prevalence of *G. lamblia*, *Cryptosporidium* sp., and *E. histolytica* infection among children with diarrhoea at PML, Accra

4.1.1.1 Prevalence of *G. lamblia* and *Cryptosporidium* sp. among diarrhoeic and non-diarrhoeic cases

Table 4.1 represents the parasitological results of stools from both children with and without diarrhoea. The results show that there were both *G. lamblia* and *Cryptosporidium* sp. infections among the children. The only other parasite detected in the stool samples in addition to the two mentioned parasites was *Entamoeba histolytica*, found in only 3 out of 356 diarrhoeal cases, giving a low prevalence of 0.8% (Fig. 4.1). Among children with diarrhoea, 21 out of 365 were positive for *G. lamblia* infections, forming a prevalence rate of 5.8%. Also, 81 out of the total (i.e. 365 diarrhoeal cases) were positive for *Cryptosporidium* sp., giving a prevalence rate of 22.2%. None of the non diarrhoeal cases had *E. histolytica* infections but both *G. lamblia* and *Cryptosporidium* sp. were present in relatively lower

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prevalence rates compared with the symptomatic infections. There was a statistically significant difference in *Cryptosporidium* sp. infections between children with diarrhoea and those without ($P < 0.0001$), as well as for *G. lamblia* infections among the same two categories of children ($P < 0.0001$). Rate of co-infections of the two parasites among the children with diarrhoea, was recorded to be low, that is 3 out of 365 cases (0.8%).

Table 4.1 Parasite distribution among 365 diarrhoeic and 120 non-diarrhoeic children at the PML hospital, Accra, Ghana

Parasite	Number and Percentage (%) infected			P- value
	Symptomatic **n** (%)	Asymptomatic **n** (%)	Total, **n** (%)	
G. lamblia	21 (5.8)	6 (5.0)	27 (5.6)	P< 0.0001
Cryptosporidium sp.	81 (22.2)	4 (3.3)	85 (17.5)	P<0.0001
E. histolytica	3 (0.8)	0 (0.0)	3 (0.6)	P=0.0143

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4.1.1.2 Risk factors associated with *G. lamblia* and *Cryptosporidium* sp. infection among children with diarrhoea

The risk factors suspected to be associated with *G. lamblia* and *Cryptosporidium* sp. were investigated among children with diarrhoea only. These factors included sex and age of child, educational background of mother, source of drinking water for the child, breastfeeding habits of mother, presence of animals at home, source of food for the child, and seasonality.

4.1.1.2.1 Sex distribution of *G. lamblia* and *Cryptosporidium* sp. infections

In all, the number of males who participated in the study was 211, whilst females were 154. For *G. lamblia* infections, 15 of males (i.e. 7.1%) were infected, whilst 6 of the females, also forming 3.9% were infected. The results show that differences of infection of *G. lamblia* among the two sexes was not statistically significant (P >0.05).

Table 4.2 Odds ratios and 95% confidence intervals for the association of *G. lamblia* infection and sex of child

Gender	No	No Infected	% Infected	X^2	P-value	OR	95% C.I.
Males	211	15	7.1				
Females	154	6	3.9	1.693	0.193	1.888	0.715 -4.982
Total	365	21	5.8				

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Table 4.3 Odds ratios and 95% confidence intervals for the association of *Cryptosporidium* infection and sex of child

Gender	No	No Infected	% Infected	X^2	P-value	OR	95% C.I.
Males	211	46	21.8				
Females	154	35	22.7	0.044	0.883	0.948	0.576 - 1.561
Total	365	81	22.2				

Similarly, 46 males out of the total males (i.e. 21.8%) had *Cryptosporidium* sp. infection, whilst 35 of the females, also forming, 22.2% were infected. The difference in infection was statistically insignificant (P >0.05). These observations imply that sex is not a risk factor for any of the two parasites.

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4.1.1.2.2 Age distribution of *G. lamblia* and *Cryptosporidium* sp. infections

The study was conducted among children of age 5 years and below. The results show that the highest rate of infection of *G. lamblia* parasite (11.1%) occurred among children of age group 2-3 years. Infection was generally low for children of age one (1) year and below (4.6%). Infection rates of *G. lamblia* between toddlers and older children was however not statistically significant (P=0.729).

Table 4.4 Odds ratios and 95% confidence intervals for the association of *G. lamblia* infection and age of child

Age group	No	No Infected	% Infected	X^2	P-value	OR	95% C.I.
< 6 months	74	3	4.1			1.893	0.675-1.956
6-12 months	165	8	4.8			1.914	0.564-2.432
13-24 months	107	8	7.5	2.038	0.729	2.824	0.432-2.854
25-36 months	9	1	11.1			3.456	0.325-3.654
37-48 months	10	1	10			2.377	0.435-2.547
Total	365	21	5.8				

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Table 4.5 Odds ratios and 95% confidence intervals for the association of *Cryptosporidium* infection and age of child

Age group	No	No Infected	% Infected	X^2	P-value	OR	95% C.I.
< 6 months	74	13	17.6			2.856	1.672-3.042 *
6-12 months	165	49	29.7			3.111	1.973-3.201 *
13-24 months	107	16	15	13.043	0.011	2.050	0.254-2.761
25-36 months	9	3	33.3			3.432	1.675-3.456*
37-48 months	10	0	0			-	
Total	365	81	22.2				

***Significant at 5%**

For *Cryptosporidium* infections, although high prevalence rates occurred among children of ages one year and below, children of age group 2-3 years recorded the highest rate of infection. There was no infection of the parasite among children aged more than 3 years.

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4.1.1.2.3 Educational background of mothers

The study revealed that although a relatively high *Cryptosporidium* infection rate (33.3%) occurred among children whose mothers had no formal education, no specific trend was observed, as lower infection rate was comparatively realized for mothers with Primary education (15%) than those with Secondary (29.7%) or Tertiary(17.6%) levels of education (Table 4.6).

Table 4.6 Odds ratios and 95% confidence intervals for the association of *G. lamblia* infection and selected mother's educational background

Education	No	No Infected	% Infected	X^2	P-value	OR	95% C.I.
Tertiary	28	2	7.1			0.733	0.234-1.035
Secondary	250	16	6.4			0.914	0.543-1.543
Primary	40	1	2.5	1.264	0.732	1.232	0.876-1.546
None	47	2	4.3			2.548	0.453-2.749
Total	365	21	5.8				

Similar observations were made for *G. lamblia* infections in which no specific trend was recorded (Table 4.6). It appears therefore that in this study, the level of maternal education does not influence infection of the two parasites among the children.

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Table 4.7 Odds ratios and 95% confidence intervals for the association of *Crytosporidium* infection and mother's educational background

Education	No	No Infected	% Infected	X^2	P-value	OR	95% C.I.
Tertiary	28	13	17.6			0.926	0.654-1.453
Secondary	250	49	29.7			2.437	1.768-2.763*
Primary	40	16	15	13.043	0.011	1.232	1.108-2.319*
None	47	3	33.3			3.629	2.456-3.896
Total	365	0	0				

*Significant at 5%

4.1.1.2.4 Source of drinking water for child

Regarding source of drinking water for the children, there were three categories, namely, children who depended solely on Sachet (bagged) water, Pipe borne water, and those who depended on both sources (Table 4.8 & 4.9). The results show that predominantly more children depended on Sachet water (337) than the other sources (i.e. 26 and 2 for Pipe borne and both sources, respectively). *G. lamblia* infections occurred among 20 out of the children who depended on Sachet (i.e. 5.9%), whilst only 1 child out of those who depended on Pipe borne water (i.e. 3.8%) was infected. Also, 78 children out of those who depended on Sachet water (i.e. 23.1% were infected with *Cryptosporidium* sp., whilst 3 (11.5%) of the children who also depended on Pipe borne were infected with *Cryptosporidium* sp. Clearly, significantly more infections of both parasites were associated with Sachet water than Pipe-water.

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Table 4.8 Odds ratios and 95% confidence intervals for the association of *G. lamblia* infection and source of drinking water

Water	No	No Infected	% Infected	X^2	P-value	OR	95% C.I.
Sachet	337	20	5.9			1.645	0.543-1.246
Pipe borne	26	1	3.8	0.317	0.853	0.986	0.234-1.429
Both	2	0	0			1.000	0.982-1.023
Total	365	21	5.8				

Table 4.9 Odds ratios and 95% confidence intervals for the association of *Cryptosporidium* infection and source of drinking water

Water	No	No Infected	% Infected	X^2	P-value	OR	95% C.I.
Sachet	337	78	23.1			1.935	0.763-2.176
Pipe borne	26	3	11.5	2.457	0.293	0.725	0.564-1.342
Both	2	0	0			1.000	0.895-1.241
Total	365	81	22.2				

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4.1.1.2.5 Effects of Breastfeeding habits on *G. lamblia* and *Cryptosporidium* sp infections

Breastfeeding could be done for exclusively 6 months or not. Reports from this study indicate that, in all 218 mothers practiced exclusive breastfeeding whilst 131 did not, and there was no response for 16 mothers (Tables 4.10 & 4.11). Twelve (12) out of those who breastfed exclusively, that is, 5.5%, had their children infected with *G. lamblia*. Similarly, 6 of mothers who did not practice exclusive breastfeeding had their children infected with the parasite, and the difference was not statistically significant (P> 0.05).

Table 4.10 Odds ratios and 95% confidence intervals for the association of *G. lamblia* infection and breastfeeding habits

Breast Feeding	No	No Infected	% Infected	X^2	P-value	OR	95% C.I.
Exclusive	218	12	5.5			3.148	0.983-3.276
Not Exclusive	131	6	4.6	5.342	0.069	2.827	0.782-2.901
NR	16	3	18.8			1.529	0.916-1.876
Total	365	21	5.8				

With regards to *Cryptosporidium* sp. infections, 47 out of mothers who practiced exclusive breastfeeding (21.6%) had their children infected, whilst 31 (i.e. 23.7%) of those who did not practice exclusive breastfeeding had their children infected with the parasite. The difference was not statistically significant (P> 0.05), suggesting that breastfeeding habits did not have influence on the rate of the infection of these parasites among the children.

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Table 4.11 Odds ratios and 95% confidence intervals for the association of *Cryptosporidium* infection and breastfeeding habits

Breast Feeding	No	No Infected	% Infected	X^2	P-value	OR	95% C.I.
Exclusive	218	47	21.6			3.148	0.567-3.344
Not Exclusive	131	31	23.7	0.325	0.85	2.827	0.679-3.129
NR	16	3	18.8			1.724	0.768-1.881
Total	365	81	22.2				

4.1.1.2.6 Effects of source of food on *G. lamblia* and *Cryptosporidium* sp. infections

There were three categories of children regarding their source of food, which include, those whose food was prepared at home only, those whose food was obtained from the street only, and those whose food was from both sources (Tables 4.12 & 4.13). The study revealed that, majority of the children had their food prepared at home (i.e. 217 out of 365), whilst a relatively small number (10), had their food purchased on the street. An appreciably high number (110) also depended on food from both sources. Children who depended on food from both sources had the lowest infection of *G. lamblia* (3.6%). Children who were fed with street food only had no infection of *Cryptosporidium* sp. detected in them.

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Table 4.12 Odds ratios and 95% confidence intervals for the association of *G. lamblia* infection and source of food

Source of Food	No	No Infected	% Infected	X^2	P-value	OR	95% C.I.
Home	217	13	6			0.905	0.675-1.456
Street	10	1	10			2.886	0.564-2.976
Both	110	4	3.6	2.535	0.469	1.853	0.692-2.012
NR	28	3	10.7			0.543	0.342-1.398
Total	365	21	22.2				

Table 4.13 Odds ratios and 95% confidence intervals for the association of *Cryptosporidium* sp. infection and source of food

Source of Food	No	No Infected	% Infected	X^2	P-value	OR	95% C.I.
Home	217	39	18			0.611	0.453-1.760
Street	10	0	0			1.513	1.271-2.386*
Both	110	36	32.7	12.174	0.007	1.967	1.456-2.692*
NR	28	6	21.4			1.456	1.245-2.199*
Total	365	81	22.2				

Generally, food prepared at home did not offer enough protection of the children from both parasites, as infection rates of 6% and 18% for *G. lamblia* and *Cryptosporidium* sp. respectively were observed.

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4.1.1.2.7 Presence of domestic animals and *G. lamblia* or *Cryptosporidium* sp. infections

In the present study, there were more children without domestic animals at home (209) than those who had them (156) (Tables 4.14 & 4.15).

Table 4.14 Odds ratios and 95% confidence intervals for the association of *G. lamblia* infection and presence of domestic animals

Animals	No	No Infected	% Infected	X^2	P-value	OR	95% C.I.
Yes	156	9	5.8				
No	209	12	5.7	0.001	0.999	1	0.413 - 2.448
Total	365	21	5.8				

From those who had domestic animals at home, 9 (i.e. 5.8%) were infected with *G. lamblia*, whilst 41 out of those who did not have (i.e. 5.7%) were also infected by the parasite. The difference is statistically insignificant (P> 0.05).

Table 4.15 Odds ratios and 95% confidence intervals for the association of *Cryptosporidium* infection and presence of domestic animals

Animals	No	No Infected	% Infected	X^2	P-value	OR	95% C.I.
Yes	156	40	25.6				
No	209	41	19.6	1.877	0.171	1.433	0.861 -2.320
Total	365	81	22.2				

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Forty (40) children out of those who had domestic animals at home (25.6%) were reported to have *Cryptosporidium* infections, whilst 41 of those who did not have (i.e. 19.6%) were infected with *Cryptosporidium* sp. The difference was not statistically significant (P> 0.05). The significance of these observations is that, having domestic animals at home does not pose higher risk of infection of the two parasites to children than when there are no animals in the home.

4.1.1.2.8 Seasonality and *G. lamblia* or *Cryptosporidium* sp. infections

Although the two parasites were present among the children for each month throughout the year (Fig. 4.2), effects of seasonality was obtained by comparing total number of infections of each parasite within the rainy season, and the dry season. In all, there were 189 diarrhoeal cases in the dry season, and 176 in the rainy season (Tables 4.16 & 4.17).

The reports from the study indicate that there were 13 *G. lamblia* infections (6.9%) in dry season, and 8 (4.5%) during the rainy season. Similarly, *Cryptosporidium* infections in the dry season was 37 (19.6%), and 44(25%) during the rainy season. The observations made indicate that none of the parasites has a specific preference for any of the seasons, as differences between seasonal occurrence were not significant (P=0.33, OR=1.551, 95%CI=0.627-3.037, and P=0.213, OR=0.73, 95%CI=0.443-1.199, for *G. lamblia* and *Cryptosporidium* sp. respectively).

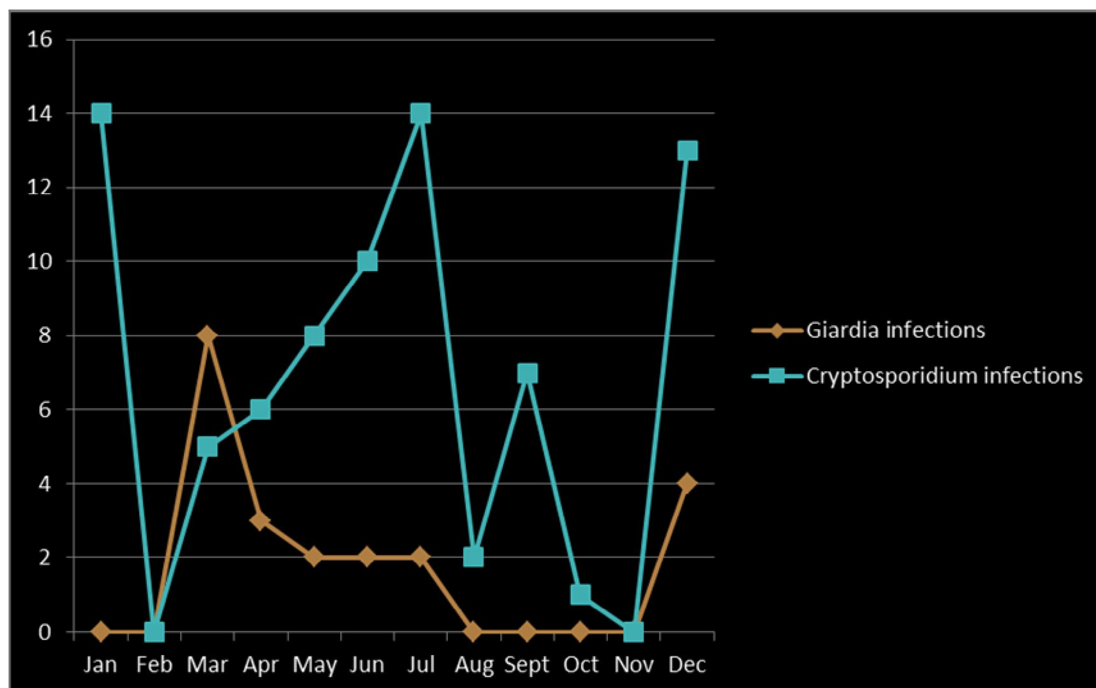

Figure 4.2 Monthly distributions of *G. lamblia* and *Cryptosporidium* sp. infections among children with acute diarrhoea at PML, Accra

Table 4.16 Odds ratios and 95% confidence intervals for the association of *G. lamblia* infection across seasons

Season	No	No Infected	% Infected	X^2	P-value	OR	95% C.I.
Dry	189	13	6.9				
Rainy	176	8	4.5	0.915	0.33	1.551	0.627 - 3.037
Total	365	21	5.8				

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Table 4.17 Odds ratios and 95% confidence intervals for the association of *Cryptosporidium* infection across seasons

Season	No	No Infected	% Infected	X²	P-value	OR	95% C.I.
Dry	189	37	19.6				
Rainy	176	44	25	1.553	0.213	0.73	0.443 - 1.199
Total	365	81	22.2				

4.1.2 Community-based study

A total of 119 children were studied in 3 selected daycare centres at the Korle Gonno community, Accra. They comprised of 58 males and 61 females of ages 5 years and below. None of the children enrolled in the study from these selected centres reported of any symptoms of diarrhoea or other related clinical signs of gastroenteritis such as vomiting, fever, abdominal pain and malaise. The study revealed that both *G. lamblia* and *Cryptosporidium* sp. were present in all the daycare centres (Table 4.18).

Table 4.18 Distribution of *G. lamblia* and *Cryptosporidium sp*. parasites in 3 daycare centres at Korle Gonno community, Accra.

	Number and percentage prevalence		
Types of parasites	**Daycare centres**		
	Daycare I N= 42 (%)	Daycare II N= 37 (%)	Daycare III N= 40 (%)
G. lamblia	4 (9.5)	3 (8.1)	5 (12.5)
***Cryptosporidium* sp**	1 (2.4)	2 (5.4)	2 (5.0)

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4.1.2.1 Prevalence of *G. lamblia* and *Cryptosporidium* sp.

The overall prevalence of *G. lamblia* infections among the children in all three daycare centres was 10.1% (i.e. 12 out of 119) whilst that of *Cryptosporidium* sp. was 4.2% (i.e. 5 out of 119).

4.1.2.2 Sex distribution of *G. lamblia* and *Cryptosporidium* sp.

The results also indicate that infection of both parasites were more in males than in females (Fig. 4.3). For *G. lamblia*, there were 7 out of 58 male children (i.e. 14.4%), as compared with 5 out of 61 female children (i.e. 9.8%) infected. Similarly, 3 out 58 males (i.e. 6.2%) were infected with *Cryptosporidium* sp., whereas 2 out of 61 females (i.e. 3.9%) were infected with the same parasite. However, the differences in rates of infection for these parasites among the sexes were not statistically significant (P=0.69 and P= 0.95 for *G. lamblia* and *Cryptosporidium* sp. infections respectively) (Table 4.19 & 4.20).

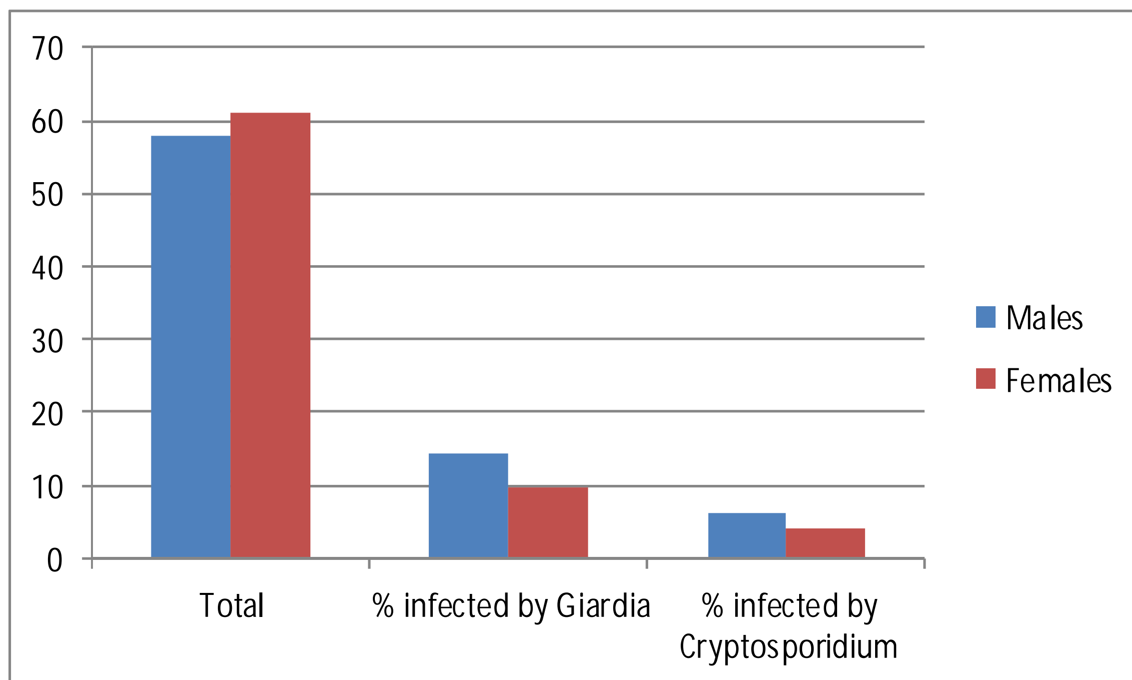

Fig. 4.3 Gender distribution of children enrolled in study and proportions (%) infected with *Giardia* and *Cryptosporidium* sp in selected daycare centres at Korle Gonno community, Accra

Table 4.19 Gender distribution of *G. lamblia* infection among children in the selected daycare centres at Korle Gonno, Accra.

Sex	No. of children investigated	*Giardia* positive (%)	*Giardia* negative (%)	P-value
Males	58	7 (14.4)	51 (85.6)	0.69
Females	61	5 (9.8)	56 (90.2)	

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Table 4.20 Gender distribution of *Cryptosporidium* sp. infection among children in the selected daycare centres at Korle Gonno, Accra.

Sex	No. of children investigated	*Cryptosporidium* positive (%)	*Cryptosporidium* negative (%)	P-value
Males	58	3 (6.2)	55 (93.8)	P= 0.95
Females	61	2 (3.9)	59 (96.1)	

Table 4.21 Age distribution of *G. lamblia* and *Cryptosporidium* sp. infections among children at daycare centres at Korle Gonno, Accra.

Age group (months)	*G. lamblia*		*Cryptosporidium* sp.	
	No. examined	No. positive (%)	No. examined	No. positive (%)
< 6 (babies)	5	0 (0)	5	0 (0)
6 -12 (toddlers)	12	0 (0)	12	0 (0)
> 12- 24	25	1 (4)	25	1 (4)
> 24- 30	27	0 (0)	27	1 (3.7)
> 30 -36	14	2 (14)	14	0 (0)
> 36 -42	10	2 (20)	10	1 (10)
> 42- 48	8	2 (25)	8	1 (12.5)
> 48- 54	4	2 (50)	4	1 (25)
> 54 -60	14	3 (21)	14	0 (0)
Total	119		119	

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4.1.2.3 Age of children

As indicated in Table 4.21, the highest prevalence of *G. lamblia* occurred within the age group >48-54 months, in which 2 out of 4, forming 50% of all children in that age group who were infected. The lowest infection was within the age group >12-24 in which only one (1) child out of 25 in this age group, forming 4% was observed. None of the children below 12 months was infected with *G. lamblia*. Similarly, the age group >(24-30) recorded no *G. lamblia* infection. With regards to *Cryptosporidium* sp., the highest infection rate occurred also within age group >48-54 months, in which 1 out 4 in this age category, representing 25% had infection. The age group, >42-48 also recorded 1 infection out 8 children in this group which forms 12.5%. The lowest infection occurred in >24-30 age group, in which 1 out 27 (i.e. 3.7%) was observed. Other observations made were that, the age groups, 12 months and below, >30-36 months, and >54-60 months, recorded no infection of *Cryptosporidium* sp.

4.1.2.4 Breastfeeding habits

The results obtained from screening children who were exclusively breastfed and those that were not exclusively breastfed, for *G. lamblia* and *Cryptosporidium* sp. infections have been presented in Figs. 4.4 & 4.5. In the two categories of breastfeeding habits, 111 children were exclusively breastfed whilst only 8 children were not. Regarding the rate of parasites infection, 8 out of children exclusively breastfed (i.e. 7.2%) had *G. lamblia* infections, whilst 4 out all children who were not exclusively breastfed, representing 50% of those children were also infected with *G. lamblia.* It was also observed that only 1 child out of those who were exclusively breastfed (i.e. 0.9%) was infected with *Cryptosporidium* sp. However, infection of this parasite among children who were not breastfed exclusively was as high as

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50% (i.e. 4 out of 8 children). Exclusive breastfeeding appears to offer some protection against the two parasites

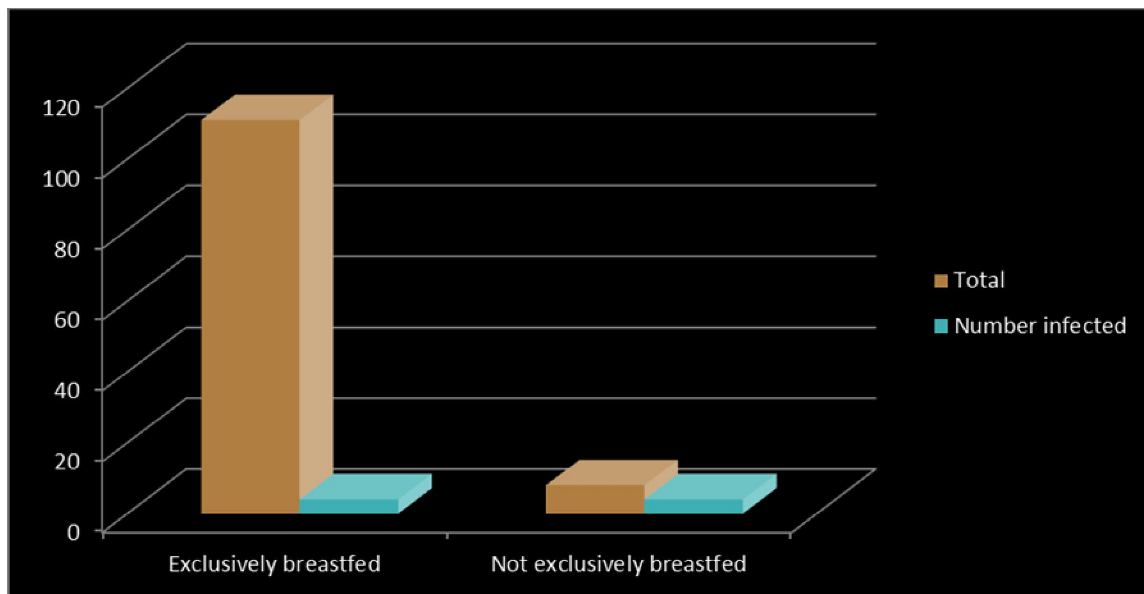

Fig 4.4 *G. lamblia* infections among children who were exclusively breastfed and children who were not ecxlusively breastfed.

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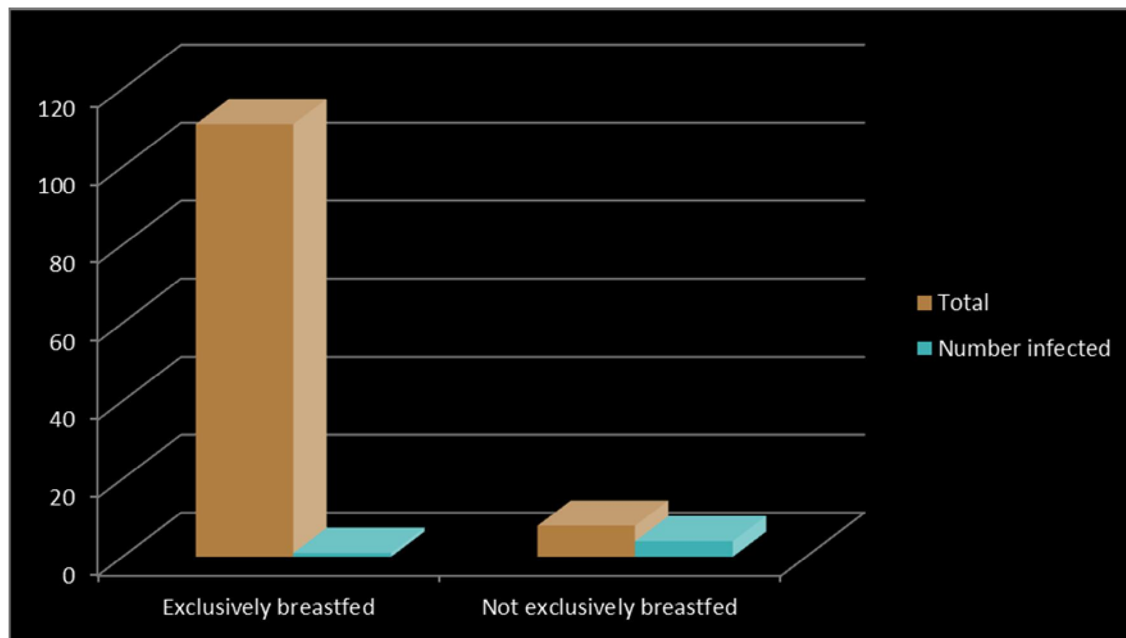

Fig. 4.5 *Cryptosporidium* sp. infections among children who were exclusively breastfed and children who were not exclusively breastfed.

4.2 Detection and genotyping of *G. lamblia* and *Cryptosporidium* sp. isolates

The molecular detection and genotyping of *G. lamblia* and *Cryptosporidium* sp. in the stool samples were achieved through the Polymerase chain reaction (PCR) and the Restriction Fragment Length Polymorphism (RFLP) respectively. The isolates were also sequenced to confirm genotyping results by RFLP.

4.2.1 PCR analysis

The results of PCR analysis confirmed the positivity of samples which tested positive by either microscopy or enzyme immunoassay test.

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4.2.1.1 NESTED PCR analysis for *G. lamblia* and *Cryptosporidium* sp.

The isolates of *G. lamblia* and *Cryptosporidium* sp. that were used for the molecular analysis (shown in Appendix 4) were obtained from the epidemiological study at the hospital (PML), which forms part of the initial aspect of the entire study. The samples were in two categories, namely samples collected from children with diarrhoea, and samples from non-diarrhoeal children, serving as a control group. The results obtained show that samples which tested positive for *G. lamblia* by enzyme immunoassays (27), during the epidemiological study also tested positive by PCR, showing an amplification of 500bp of the glutamate dehydrogenase gene (gdh) of *G. lamblia* (Fig. 4.6). Similarly, from a total of 85 samples which tested positive for *Cryptosporidium* sp. by enzyme immunoassays, 72 tested positive by PCR showing amplification of 450bp of 18S rRNA gene of *Cryptosporidium* sp. (Fig. 4.8). The samples used for molecular analysis were from the hospital-based study alone. Community study samples were not analysed by PCR as a result of some challenges of poor storage conditions encountered during the period of sampling. The samples were not included on the basis that, poor storage conditions had caused a probable degradation of the DNA of the parasites in the stool samples, as a result of which PCR amplification and gel electrophoresis did not show any bands apart from the control lanes.

4.2.1.2. PCR-RFLP analysis of *G. lamblia*

The purpose for performing the PCR-RFLP analysis was to identify the genotypes (or assemblages) to which the positive isolates (listed in Appendix 4) belong. To do this, a 461bp fragment of *gdh* was amplified in the second round of PCR using GDHeF and GDHiR for all the isolates which were positive for *G. lamblia*, and the products of amplification further digested by NlaIV restriction enzyme. The pattern of bands obtained after gel electrophoresis, shown on Fig. 4.7 indicates that all isolates in the present study were genotype (assemblage)

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B (BIII or BIV), with reference to earlier studies (described by Read *et al*., 2004 on Table 4.24). These include both symptomatic and asymptomatic infections. Figure 4.7 shows the results in which only two bands of sizes 120bp and 290bp were obtained. In the present study, the restriction enzyme, RsaI which is capable of distinguishing between assemblages BIII and BIV was not used because it was not available.

Table 4.22 Predicted fragment sizes (bp) and diagnostic profile (bp) of *G. lamblia* genetic assemblages when digested with NlaIV and RsaI (Read et al., 2004).

Assemblage	Enzyme (No of restriction sites)	Diagnostic genotyping profile
AI	NlaIV (5)	90, 120, 150
AII	NlaIV (6)	70, 80, 90, 120
BIII	NlaIV (2)	120, 290
BIV	NlaIV (2)	120, 290
C	NlaIV (4)	70, 120, 190
D	NlaIV (3)	120, 250
E	NlaIV (4)	80, 100, 220
BIII	RsaI (2)	130, 300
BIV	RsaI (1)	430

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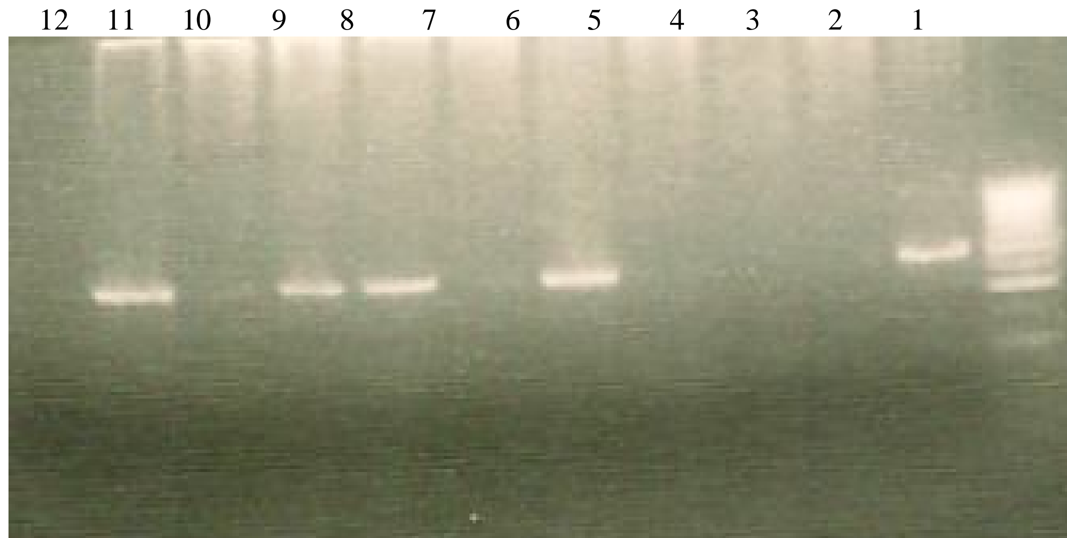

Fig 4.6 A Nested PCR at the gdh locus for identification of *G. lamblia* (Lane 1 is 100bp molecular ladder

Lanes 2-10 are patient samples. Lanes 11 and 12 are positive & negative controls respectively).

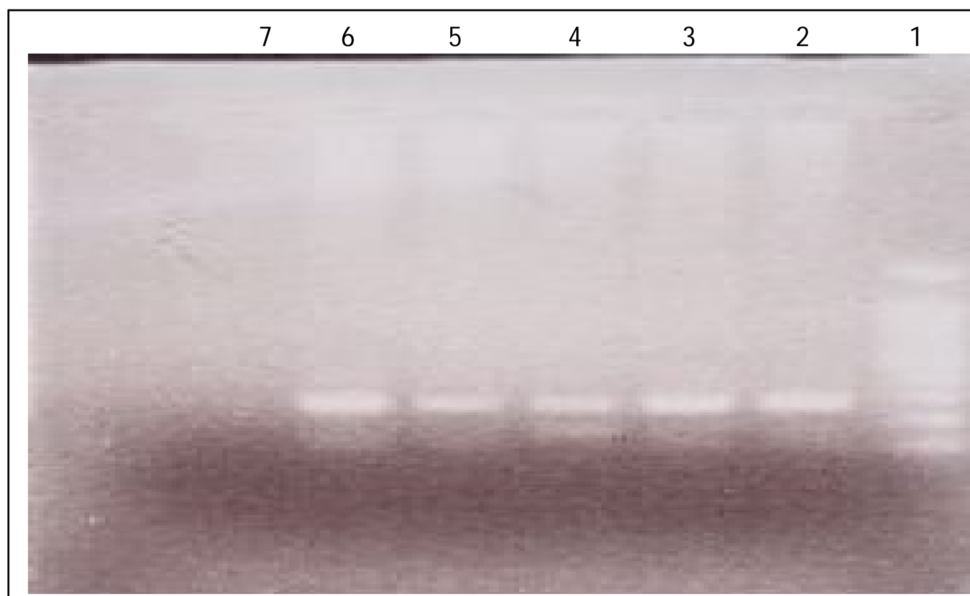

Fig 4.7 A Nested PCR-RFLP at the gdh locus with NlaIV restriction enzyme for *Giardia* assemblages/genotypes (Lane 1= 100bp molecular ladder, Lanes 2-6 are patient samples, all showing assemblage B, Lane 7 is negative control).

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4.2.1.3 PCR-RFLP analysis for *Cryptosporidium* sp.

In a similar manner as considered for *G. lamblia* isolates in the present study, the 18S rRNA PCR-RFLP analysis was also performed for species identification of *Cryptosporidium* sp. for all isolates obtained in the study. Two different restriction enzymes, namely, VspI and SspI were used to digest the PCR seondary product of *Cryptosporidium* sp. isolates simultaneously. Analysis of the amplicons (Fig. 4.9) revealed several different patterns in the profile that were suspected to be *C. muris*, *C. andersoni*, *C. parvum* and *C. hominis*. Also, the results for some isolates suggest an incomplete digestion and therefore do not match a clear specific species. Generally, analysis of the profile was done with reference to Table 4.25 (adapted from a previous study by Nichols *et al.* (2003), together with an analysis performed by a software, and shown in Appendix 6.

Table 4.23 *Cryptosporidium* sp. and genotypes determined by RFLP of the amplicon defined by CPB-DIAGR/F primers after digestion with the enzymes VspI, DraI and DdeI (Nichols et al., 2003)

Cryptosporidium spp. and genotypes(total amplicon length in bp)	Amplicon length(s) (bp) as defined with			GenBank accession number
	VspI	DraI	DdeI	
C. *parvum* 1 (438)	222,104,112	None	204,68,166	L16997
	219,104,112	None	201,68,166	L16996,AF161856
C. *parvum* 2 (435)	320,112	None	42,224,166	AF093498,AF093498,AF093497
	319,112	None	265,166	AF093496,49069
C. *muris* (432)	239,104,112	50,405	221,68,166	AF087577
C. *andersoni* (431)	212,104,112	84,344	262,166	L19068,AF093495
C. *felis* (455)	47,171,104,112	None	200,68,166	AF112574
C. *baileyi* (428)	312,112	None	264,166	AF093502
C. *maleagridis* (434)	219,104,112	None	201,68,166	AF115378
C. *serpentis* (430)				
C. *wrairi* (435)				

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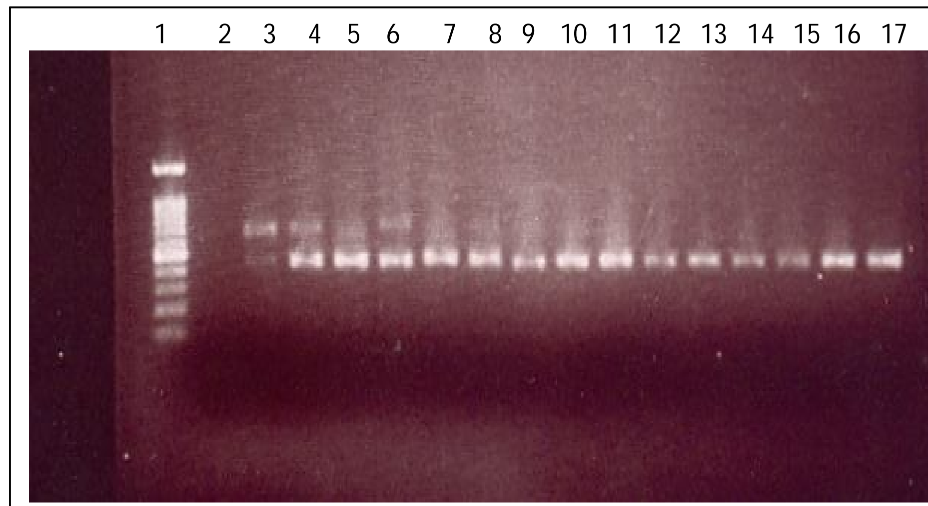

Fig 4.8 A Nested PCR at the 18S ribosomal RNA gene locus for identification of *Cryptosporidium* sp. (Lane 1 is 100bp molecular ladder Lanes 3 to 16 are patient samples. Lanes 17 and 2 are positive & negative controls respectively.

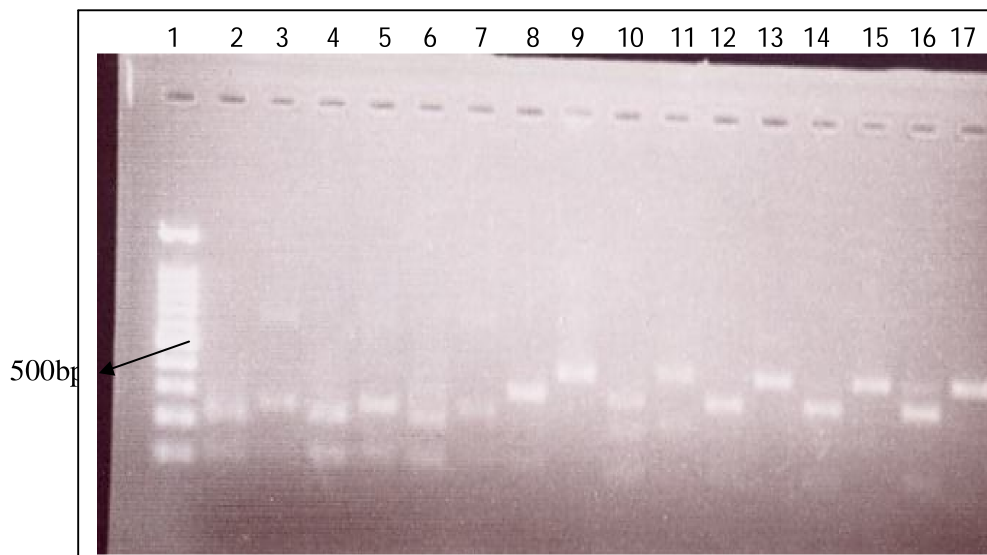

Fig. 4.9 Genotyping of Cryptosporidium isolates by RFLP analysis based on digestion of 18S rRNA gene PCR products by SspI (Lanes 3, 5, 7, 9, 11, 13, 15, 17), VspI (Lanes 2, 4, 6, 8, 10, 12, 14, 16). Lane 1= 100bp molecular marker; Lanes 1, 2 ,…..17 are all clinical samples.

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4.2.2 Nucleotide sequence analysis

In all, 10 isolates, comprising of 4 isolates of *G. lamblia* and 6 isolates of *Cryptosporidium* sp., all from the present study were sent for commercial sequencing (at Macrogen Europe Laboratory IWO, Kamer 1A3-195, Meibergdreef 39, 1105, AZ, Amsterdam, Netherlands). Results obtained from sequencing have been shown in Figs. (4.10- 4.13). The selected isolates had the following code numbers (shown in Table 4.26):

Table 4.24 Isolates of *G. lamblia* and *Cryptosporidium* sp. used for sequencing

Serial number	Isolate code	Isolate source
1	N15408	*G. lamblia* from diarrhoeal stool
2	GPML1037	*G. lamblia* from diarrhoeal stool
3	GPML983	*G. lamblia* from non-diarrhoeal stool
4	GPML1443	*G. lamblia* from non-diarrhoeal stool
5	EMO311	*Cryptosporidium* sp. from diarrhoeal stool
6	EMO346	*Cryptosporidium* sp. from diarrhoeal stool
7	EMO303	*Cryptosporidium* sp. from diarrhoeal stool
8	EMO289	*Cryptosporidium* sp. from diarrhoeal stool
9	EMO312	*Cryptosporidium* sp. from diarrhoeal stool
10	EMO325	*Cryptosporidium* sp. from non-diarrhoeal stool

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4.2.2.1 Nucleotide sequence data for *Cryptosporidium* sp.

>Emo 325_INDIAG_436

GGGGGAGGTGGTGCACGGGCTTCCGGTGATTCTATTTTTGTCATGGTATATTTCTAACACTCGTGCTTCACTGT
GCGATGCGTTAGATCATTTACTTTGAGAAAATTTGAGTGTTTCAAGCAGGCTCCCGCCTTGAAAACTTCCGCAT
GGAATAATATGATAGGACTATGGTTCTATTTAGTTGGTTTCTGGAACTAAAGTAATGATTAACAGGGACAGTT
GGGGGCATTCGTATTTAACAGCTAGAGGTGAAATTCTTAGATTTGTTAAAGACGAACTACTGCGAAAGCATTT
GCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAA
CTATAAACTATGCCGAACTAGAGATTGGGGGGTTGTCACTTCCTTACTCCCAAACCCCCCCTTAAAAAA

>Emo 312_INDIAG_434

AAAGATACCCCCGGGACCCAGGCCGCCCCACCCCCCTCCTTGCGGTGTATTTCTAACACTCGTGCTTCACTGTG
CGATGCGTTAGATCATTTACTTTGAGAAAATTTGAGTGTTTCAAGCAGGCTCCCGCCTTGAAAACTTCCGCATG
GAATAATATGATAGGACTATGGTTCTATTTAGTTGGTTTCTGGAACTAAAGTAATGATTAACAGGGACAGTTG
GGGGCATTCGTATTTAACAGCTACAGGTGAAATTCTTAGATTTGTTAAAGACGAACTACTGCGAAAGCATTTG
CCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAAC
TATAAACTATGCCGACTAGAGATTGGGGGTTGTCCCTTCCTTACTCCTCCCCCCCCCTTAAAAAACC

>Emo 289_INDIAG_439

GGGGGAAGGGTTTGCCCGTACGCGGATTCCAATTGTGTCTTCGGTTATTTCTAACACTCGTGCTTCACTGTGCG
ATGCGTTAGATCATTTACTTTGAGAAAATTTGAGTGTTTCAAGCAGGCTCCCGCCTTGAAAACTTCCGCATGGA
ATAATATGATAGGACTATGGTTCTATTTAGTTGGTTTCTGGAACTAAAGTAATGATTAACAGGGACAGTTGGG
GGCATTCGTATTTAACAGCTAGAGGTGAAATTCTTAGATTTGTTAAAGACGAACTACTGCGAAAGCATTTGCC
AAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACTA
TAAACTATGCCGACTAGAGATTGGGGGTTGTCACTTCCTTACTCTCAGCCCCCCCTTAAAAAACAAAAACAC

>Emo 303_INDIAG_434

AAGACGGGCGACGGTTCCAGGGTCCCACCACAACAATGTTATGGTATATTTCTAACACTCGTGCTTCACTGTG
CGATGCGTTAGATCATTTACTTTGAGAAAATTTGAGTGTTTCAAGCAGGCTCCCGCCTTGAAAACTTCCGCATG
GAATAATATGATAGGACTATGGTTCTATTTAGTTGGTTTCTGGAACTAAAGTAATGATTAACAGGGACAGTTG
GGGGCATTCGTATTTAACAGCTAGAGGTGAAATTCTTAGATTTGTTAAAGACGAACTACTGCGAAAGCATTTG
CCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAAC
TATAAACTATGCCGACTAGAGATTGGGGGTTGTCACTTCCTTACTCTCGGCCCCCCCTTAAAAAAAGA

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>Emo 311_INDIAG_450

AGGGAGCGAAGGTTAAAGCGCGTTTCGTGTATCTATGATCTCCTTCGCACTCTTCGGACACTCGTGCTTCACTG
TGCGATGCGTTAGATCATTTACTTTGAGAAAATTTGAGTGTTTCAAGCAGGCTCCCGCCTTGAAAACTTCCGCA
TGGAATAATATGATAGGACTATGGTTCTATTTAGTTGGTTTCTGGAACTAAAGTAATGATTAACAGGGACAGT
TGGGGGCATTCGTATTTAACAGCTAGAGGTGAAATTCTTAGATTTGTTAAAGACGAACTACTGCGAAAGCATT
TGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTA
ACTATAAACTATGCCGACTAGAGATTGGGGGTTGTCACTTCCTTACTCCTAGGCCCACCTTAAAAAATGAACCC
TTAAAAAAC

>Emo 346_INDIAG_417

AGATGGGCGCCCGGTACACGGGTGCCTCCCACTCCCTATTAACATAATTCATATTACTATATATTTTAGTATAT
GAAATTTTACTTTGAGAAAATTAGAGTGCTTAAAGCAGGCATATGCCTTGAATACTCCAGCATGGAATAATATT
AAAGATTTTTATCTTTCTTATTGGTTCTAAGATAAGAATAATGATTAATAGGGACAGTTGGGGGCATTTGTATT
TAACAGTCAGAGGTGAAATTCTTAGATTTGTTAAAGACAAACTAATGCGAAAGCATTTGCCAAGGATGTTTTC
ATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACTATGGCC
AACTTAAGAGATTGGAGGTTGTTCCCTTACTCCTCAAGCCCACCCTTAA

Fig. 4.10 Nucleotide sequence data of 6 isolates of *Cryptosporidium* sp.

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C.parvum ribosomal RNA gene for 18S rRNA (pCPA931)

Sequence ID: gi|7002|emb|X64341.1|Length: 2103Number of Matches: 1

Related Information

Range 1: 682 to 983GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
396 bits(206)	1e-110	277/305(91%)	3/305(0%)	Plus/Plus

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Query  91   TTTACTTTGAGAAAATTTGAGTGTTTCAAGCAGGCTCCCGCCTTGAAAACTTCCGCATGG  150
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Sbjct  682  TTTACTTTGAGAAAATTAGAGTGCTTAAAGCAGGCAACTGCCTTGAATACTCCAGCATGG  741

Query  151  AATAATATGATAGGACTATGGTTCTATTTAGTTGGTTTCTGGAACTAAAGTAATGATTAA  210
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Sbjct  742  AATAATAAGCAAGGACTTTTGTCTTTCTTA-TTGGTT-CTAGGACAAAAGTAATGGTTAA  799

Query  211  CAGGGACAGTTGGGGGCATTCGTATTTAACAGCTAGAGGTGAAATTCTTAGATTTGTTAA  270
             |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  800  TAGGGACAGTTGGGGGCATTCGTATTTAACAGCCAGAGGTGAAATTCTTAGATTTGTTAA  859

Query  271  AGACGAACTACTGCGAAAGCATTTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTA  330
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Sbjct  860  AGACGAACTACTGCGAAAGCATTTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTA  919

Query  331  GGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACTATAAACTATGCCGAACTAGAG  390
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Sbjct  920  GGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACTATGCCG-ACTAGAG  978

Query  391  ATTGG  395
            |||||
Sbjct  979  ATTGG  983
```

Fig. 4. 11 Nucleotide sequence data of 6 isolates *Cryptosporidium* sp.

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4.2.2.2 Nucleotide sequence data for *Giardia*

>N15408_GDHe438

CCCCGGGTTGTTTGCGGTCACACGGCTACATGGTGGTCTCCGATTCAACCCTCTGTCAACCTCTCGATCCTCAA
GTTCCTCGGCTTTGAGCAGATCCTGAAGAACTCCCTTACCACGCTTCCGATGGGCGGTGGTAAGGGCGGCTCC
GACTTCGATCCTAAGGGCAAGTCGGACAACGAGGTCATGCGCTTCTGCCAGTCCTTTATGACCGAGCTCCAGA
GGCACGTCGGGGCTGACACCGACGTTCCTGCTGGCGATATTGGCGTCGGCGGTCGCGAGATTGGTTATCTGT
TTGGACAGTACAAGCGCCTCAGGAACGAGTTCACGGGCGTCCTCACGGGCAAGAACATCAAGTGGGGCGGG
TCTCTCATCAGGCCAGAGGCCACAGGGTATGGAGCTGTCTACTTCCTGGAGGAGATGTGAGGGGACAACAAC
GGA

>GPML1037_GDHe440

TTTTCCCATACCGGGCCCCGATGCCCACCCCCATATCTGCAACGACAACACCTCTGTCAGGATCTGTACCCTGA
CGTCCCTCGGCTTTGAGCAGATCCTGAAGAACTCCCTTACCACGCTTCCGATGGGCGGTGGTAAGGGCGGCTC
CGACTTCGATCCTAAGGGCAAGTCGGACAACGAGGTCATGCGCTTTTGCCAGTCCTTTATGACTGAGCTCCAG
AGGCACGTCGGGGCTGACACCGACGTTCCTGCTGGCGATATTGGCGTCGGCGGTCGCGAGATCGGTTATCTG
TTTGGACAGTACAAGCGCCTCAGGAACGAGTTCACGGGCGTCCTCACGGGCAAGAACATCAAGTGGGGCGG
GTCTCTTATCAGACCAGAGGCCACAGGGTATGGAGCTGTCTACTTCCTGGAGGAGATGTGCAAGGACAACAC
AACGG

>GPML983_GDHe204

TTTAAGGGCCGTATCTGTGGGGCTTGTAAATGTTCAACAACGACACCGGAGGCCAGGAGAAGAGCAAGGAC
AACAGTGGGCTCATTAACTTTGAGAGAGCTAGAGTGTTTCACGCCGGCTCCCGCCTTGAGGACTTCCGCATGG
AATAAT

ATGATAGGACTATGGTTCTGATTAGTTGGTTTCTGGAACTAGAGTAATGATTAA

>GPML1443_GDHe201

AAATTAGGACCGGGGGGAAACGTGCTCCAACCCCCTCCTGCGGGCACAACGCAGCGTATTCATATTCCGAGC
GGCCAATGTATCGCAAGAGCCCCCGCAAACAACGGATGCGATCATGATCCCGACTCTGCTGCGTACCCCTGAA
ACCACATGATAGGACTATTGTTCTATTTAGTTAGTTTCTGGAACTACAGTTATGCT

Fig. 4.12 Nucleotide sequence data of 4 isolates of *G. lamblia*

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Giardia intestinalis isolate D39 glutamate dehydrogenase (gdh) gene, partial cds

Sequence ID: gi|343915472|gb|JF918512.1|Length: 434Number of Matches: 1

Related Information

Range 1: 32 to 434GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
725 bits(377)	0.0	396/403(98%)	1/403(0%)	Plus/Plus

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Sbjct  32   GGTGGTCTCCGCTTCCACCCCTCTGTCAACCTCTCGATCCTCAAGTTCCTCGGCTTTGAG  91

Query  91   CAGATCCTGAAGAACTCCCTTACCACGCTTCCGATGGGCGGTGGTAAGGGCGGCTCCGAC  150
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Query  151  TTCGATCCTAAGGGCAAGTCGGACAACGAGGTCATGCGCTTCTGCCAGTCCTTTATGACC  210
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Sbjct  152  TTCGATCCTAAGGGCAAGTCGGACAACGAGGTCATGCGCTTGTGCCAGTCCTTTATGACC  211

Query  211  GAGCTCCAGAGGCACGTCGGGGCTGACACCGACGTTCCTGCTGGCGATATTGGCGTCGGC  270
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  212  GAGCTCCAGAGGCACGTCGGGGCTGACACCGACGTTCCTGCTGGCGATATTGGCGTCGGC  271

Query  271  GGTCGCGAGATTGGTTATCTGTTTGGACAGTACAAGCGCCTCAGGAACGAGTTCACGGGC  330
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Sbjct  272  GGTCGCGAGATTGGTTATCTGTTTGGACAGTACAAGCGCCTCAGGAACGAGTTCACGGGC  331

Query  331  GTCCTCACGGGCAAGAACATCAAGTGGGGCGGGTCTCTCATCAGGCCAGAGGCCACAGGG  390
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Sbjct  332  GTCCTCACGGGCAAGAACATCAAGTGGGGCGGGTCTCTCATCAGGCCAGAGGCCACAGGG  391

Query  391  TATGGAGCTGTCTACTTCCTGGAGGAGATGTGAGGGGACAACA  433
            ||||||||||||||||||||||||||||||||   ||||||||
Sbjct  392  TATGGAGCTGTCTACTTCCTGGAGGAGATGTGCAAGGACAACA  434
```

Fig. 4.13 Sequence data of 4 isolates of *G. lamblia*

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4.2.3 Phylogenetic analysis of *G. lamblia* and *Cryptosporidium* sp.

This was an important aspect of the data analysis to further designate *Giardia* isolates into genotypes, and the *Cryptosporidium* sp. into species, for confirmation of results of PCR-RFLP and other additional information.

4.2.3.1 Phylogenetic analysis of *G. lamblia*

Chromatograms and sequences were examined using MEGA 5.20 (http://www.mbio.ncsu.edu/BioEdit/page2.html), and sequences aligned by ClustalW. Two of the 4 sequence data, belonging to isolates >GPML983_GDHe204 and >GPML1443_GDHe201 were not included in the analysis because of their short band sizes obtained after sequencing. Sequence searches were conducted using BLAST (http://www.ncbi.nlm.nih.gov/blast/). A dataset was assembled containing 2 novel and unambiguous *gdh* nucleotide sequences obtained in the present study, and 21 sequences retrieved from GenBank at the National Centre for Centre for Biotechnology Information (http://www.ncbi.nlm.nih.govl) representing the diversity of *gdh* sequences. The two isolates from the present study had 98% similarity to sequences deposited in GenBank. A dotplot analysis (Fig. 4.14) of the two isolates show that they share more similarities.

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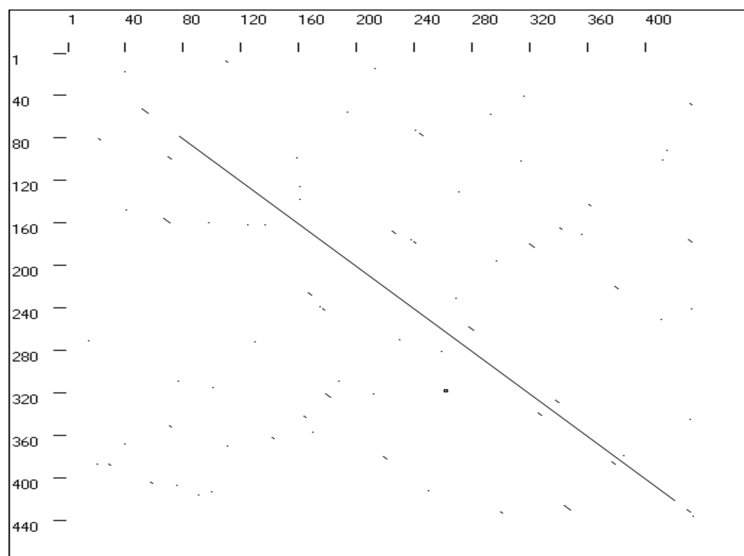

Fig. 4.14 Dotplot of the two Giardia sequences

Both isolates obtained from the present study, N15408 GDHe438 and GPML1037 GDHe440 cluster with others in GenBank (shown in Fig. 4.15) confirming the isolates as *Giardia* genotype (assemblage) B, as observed with the PCR-RFLP analysis.

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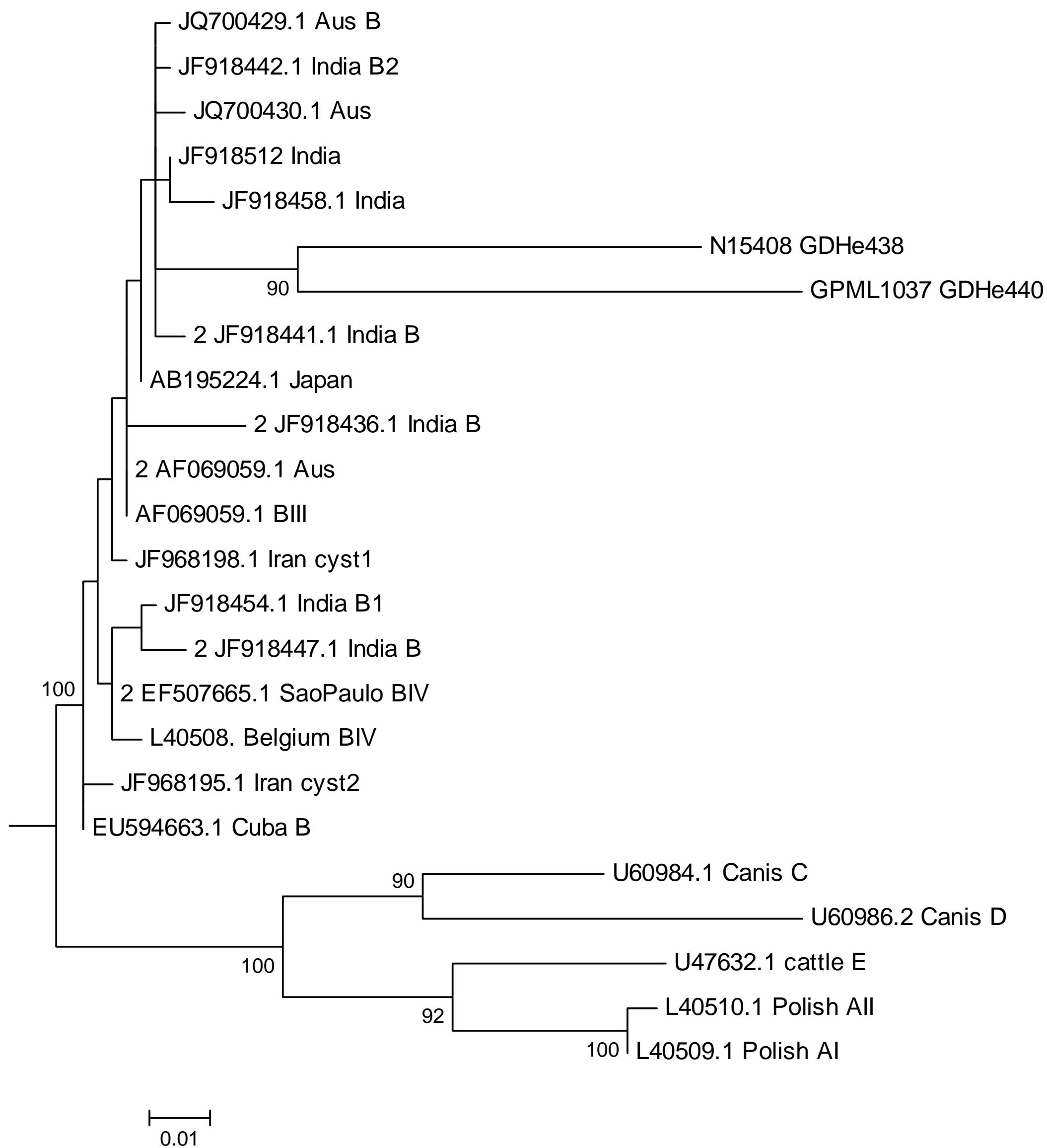

Figure 4.15 Molecular Phylogenetic analysis of *G. lamblia* genotype B from children in Accra, Ghana, with others previously deposited in GenBank by using Maximum Likelihood method of the *gdh* gene. Values on branches are percentage bootstraps values. Bootstrap values greater than 50% are shown.

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The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model (Nei *et al.*, 2000). The tree with the highest log likelihood (1599.5703) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 24 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 417 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

4.2.3.2 Phylogenetic analysis of *Cryptosporidium* sp.

The phylogenetic analysis of *Cryptosporidium* 18S rRNA gene of the six sequenced isolates were performed with methods similar to what were used for *Giardia*, with a few additions. These few additions were inclusion of a plot of similarity or identity matrix after consideration of a consensus between similar groups against any one that is different. The results from this study indicate the presence of two different species of *Cryptosporidium* among the children studied in Accra. A blast of each nucleotide sequence shows that whilst isolate EMO346 gives the best alignment (Seaview version 4.41, aligned with Clustal O) with sequences of *C. parvum* in the GenBank (E value=0, bootstrap =100), the rest, EMO312, EMO303, EMO289, EMO311, and EMO325 had the closest match with *C. andersoni*, *C. muris*, and *C. galli* (from GenBank blast). However, the phylogenetic tree analysis (Fig. 4.17) shows that they do not cluster with anyone of them. The long branched length of EMO346

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suggest that the species of that isolate is evolving faster than the others. On the similarity percentage table (Table 4.27), there were high similarities, ranging from 85.5% to 92.1% between all isolates except isolate EMO346. Isolate EMO346 alone has low similarity with the 5 others with a range of 64.9% - 69.5%.

Table 4.25 Overall similarities obtained between the *Cryptosporidium* 18S ribosomal RNA gene partial sequences

Sequences	% similarity to:					
	Emo_325	Emo_312	Emo_289	Emo_303	Emo_311	Emo_346
Emo_325		86.7	89.1	88.5	86.5	69.5
Emo_312			88.2	92.1	85.5	67.7
Emo_289				90.9	88.2	66.2
Emo_303					85.7	66.5
Emo_311						64.9
Emo_346						

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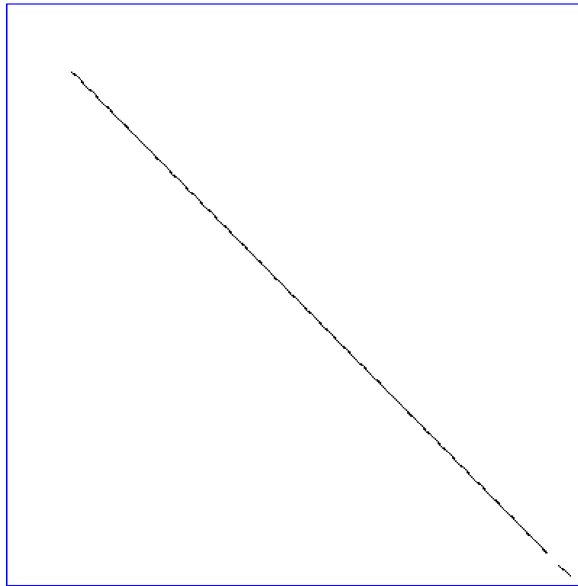
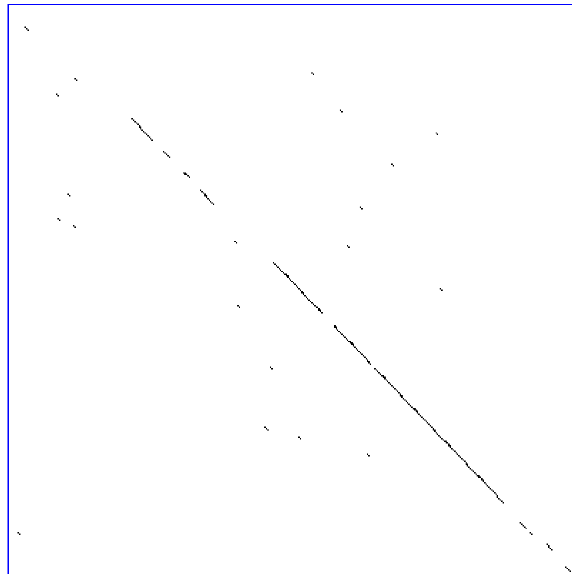


a. Dot plot of EMO_289 on horizontal axis EMO_303 on vertical axis



b. EMO_325 on horizontal axis, EMO_346 on vertical axis

Fig. 4.16 Examples of dot plot pairwise sequence comparisons obtained between the *Cryptosporidium* 18S ribosomal RNA gene partial sequences. The plotting mode was left up with the window size of 9 and mismatch limit of 1. The gap in the diagonal denotes

dissimilarity in DNA sequences. (a) Dot plot of EMO_289 and EMO_303 (b) Dot plot of EMO_325 and EMO_346

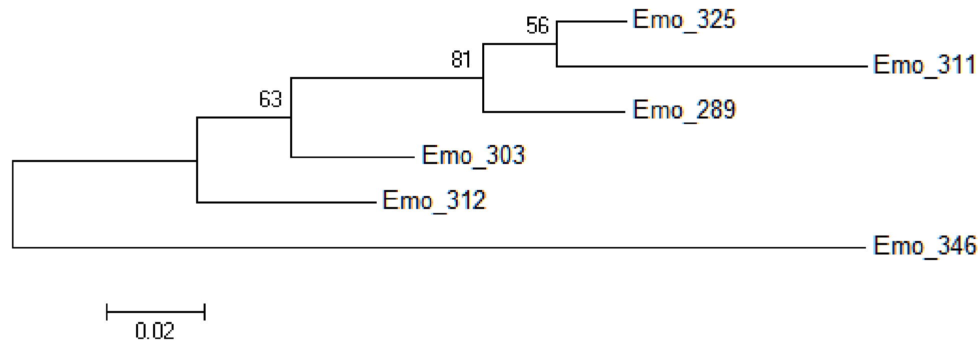


Figure 4.17 Molecular Phylogenetic anaylsis by Maximum Likelihood method

Unrooted phylogenetic tree constructed by the Maximum Likelihood method, showing the phylogenetic relationships of *Cryptosporidium* 18S ribosomal RNA gene partial sequences sequences. Bootstrap values (expressed as percentages of 500 replications) are given at the branch points.

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Nei *et al.,* 2000). The tree with the highest log likelihood (-1343.6708) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 6

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nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 413 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

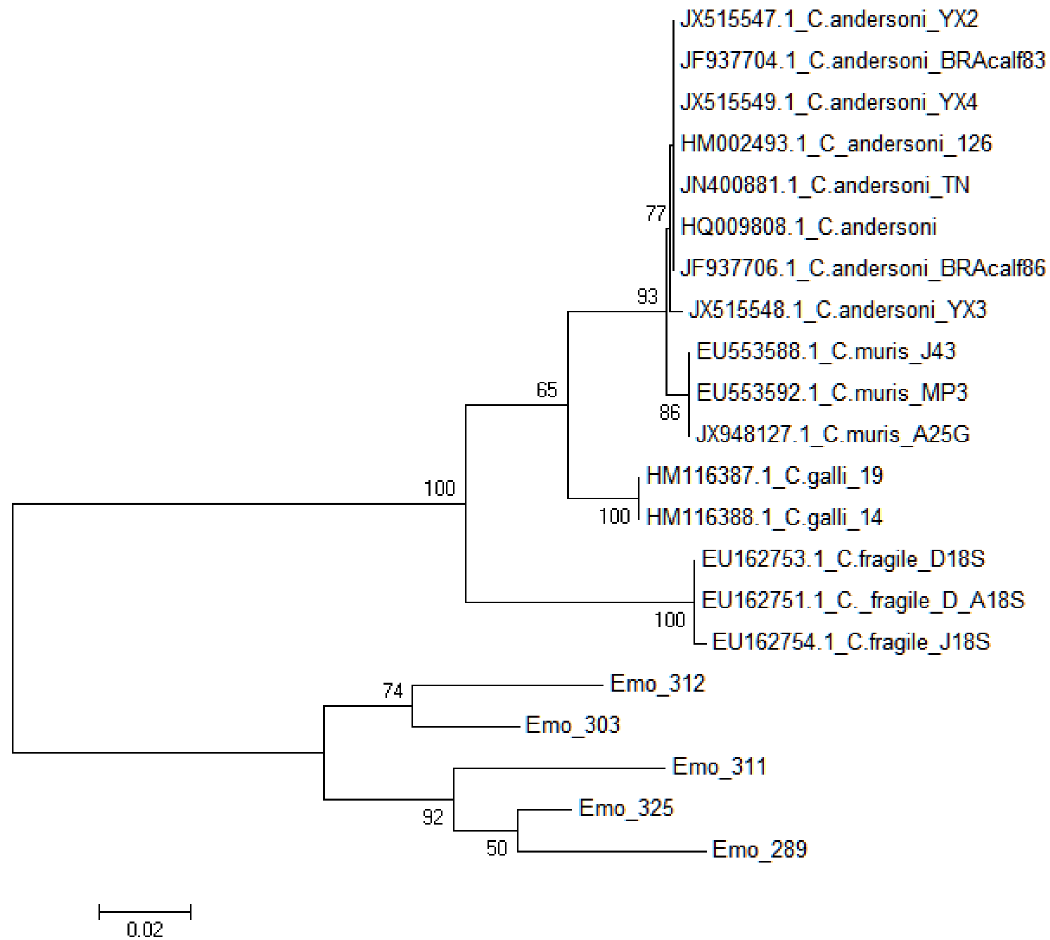

Fig. 4.18 Phylogram (maximum likelihood) of the *Cryptosporidium* 18S ribosomal RNA gene partial sequences and selected members of the *Cryptosporidium* species inferred from 18S rDNA sequence comparisons. Bootstrap values (within branches at relevant nodes) are reported when equal or greater than 40%.

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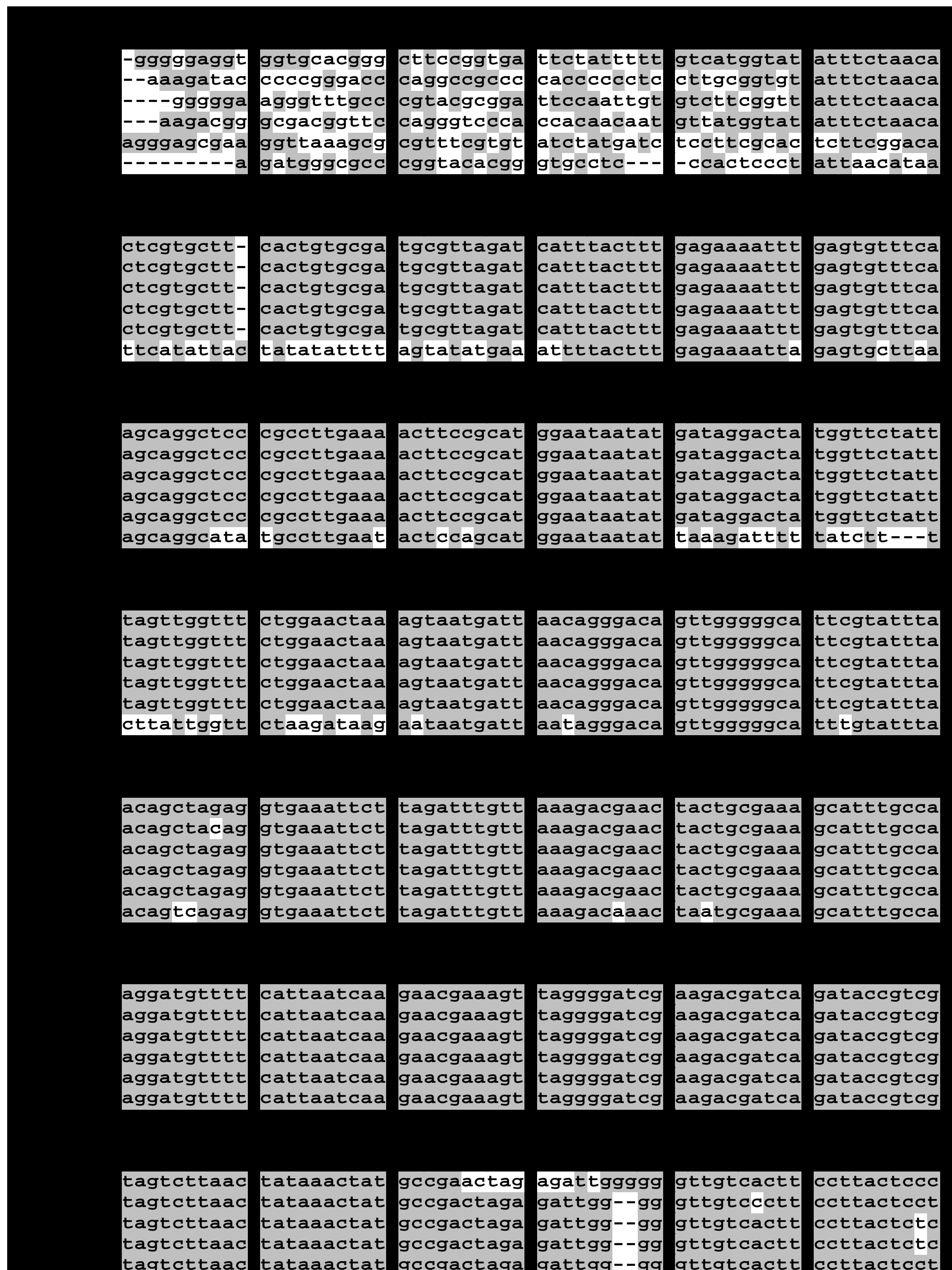

Fig. 4.19 Multiple sequence alignment of the *Cryptosporidium* 18S ribosomal RNA gene partial sequences. Conserved regions across all regions are shown in grey boxes.

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The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model (Nei *et al.*, 2000). The tree with the highest log likelihood (-1459.6135) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 21 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 395 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011)

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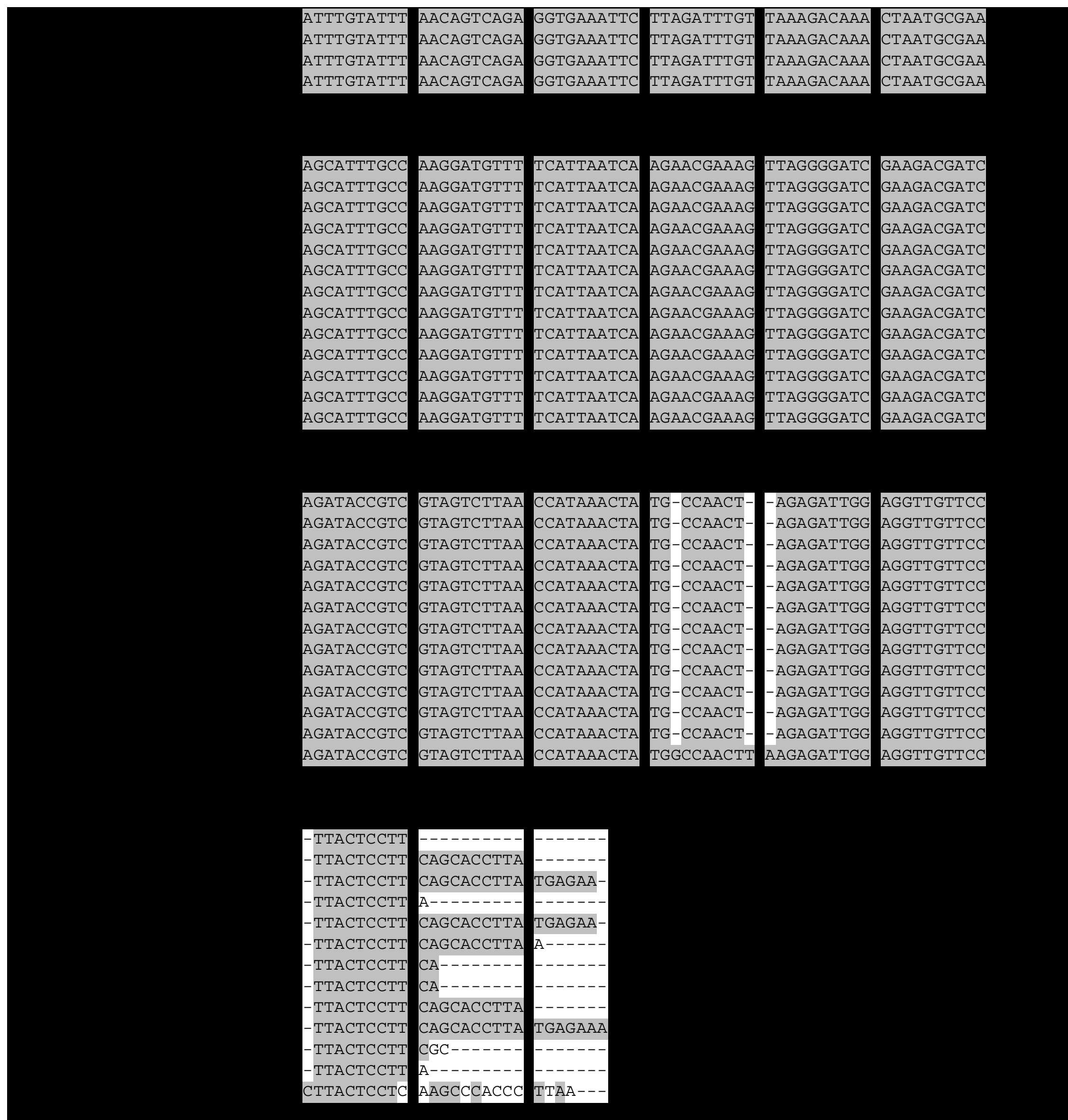

Fig. 4.20 Sequence alignments of the most homologous 18S rRNA sequences from DNA databases with EMO_346. Conserved regions across all regions are shown in grey boxes.

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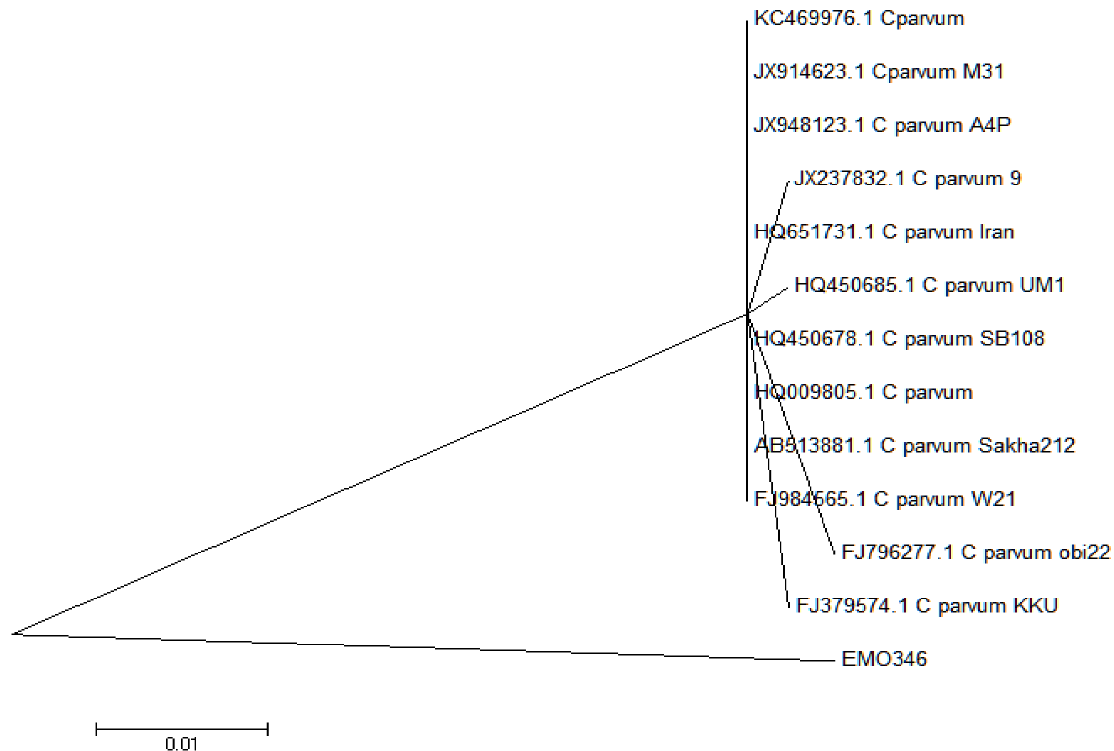

Figure 4.21 Phylogram (maximum likelihood) of the EMO_346 18S ribosomal RNA gene partial sequences and selected members of the *Cryptosporidium parvum* species inferred from 18S rDNA sequence comparisons. Bootstrap values (within branches at relevant nodes) are reported when equal or greater than 40%.

The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model (Nei *et al.*, 2000). The tree with the highest log likelihood (-784.7519) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of

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pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 13 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 447 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011).

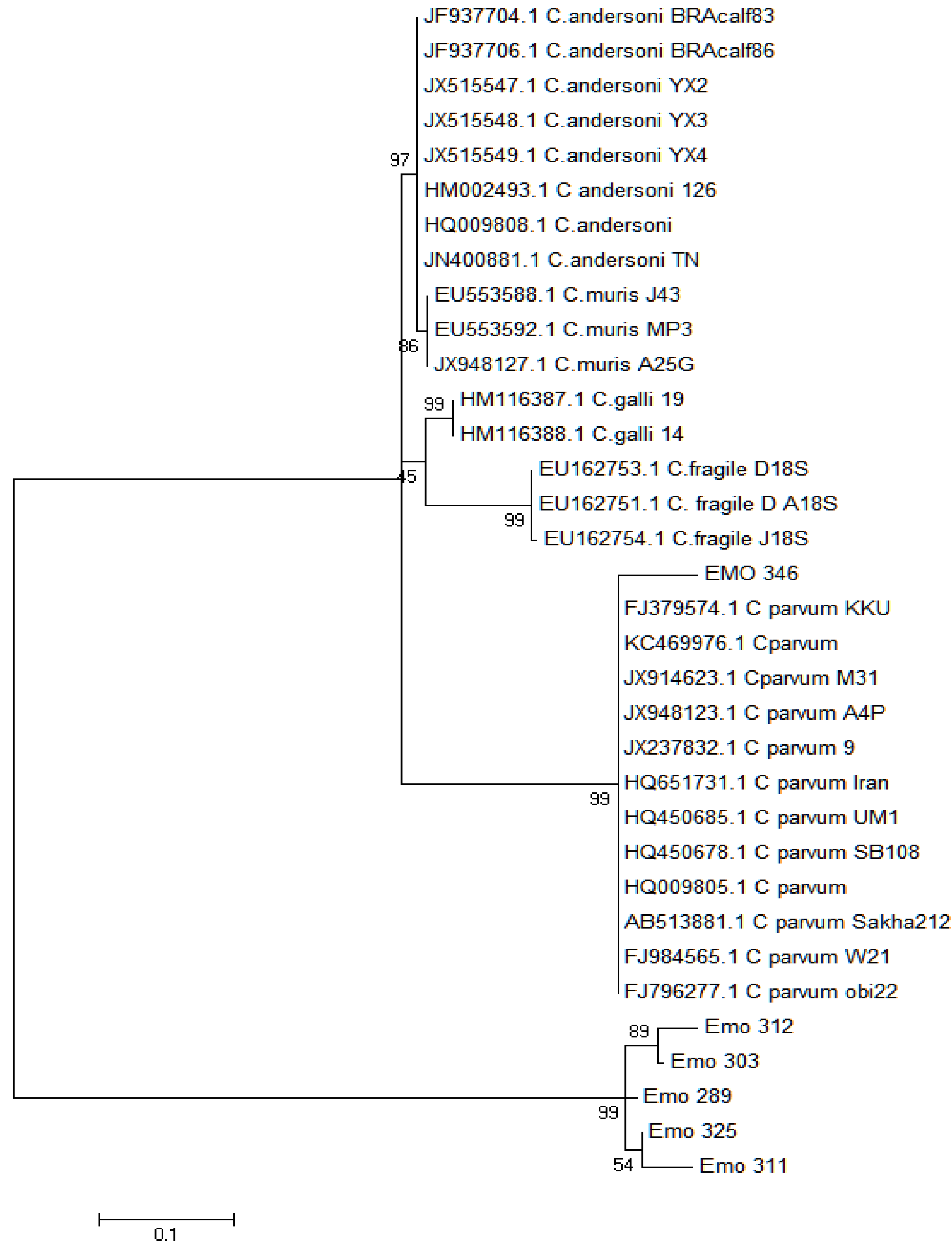

Fig 4.22 Phylogram (maximum likelihood) of the all *Cryptosporidium* 18S ribosomal RNA gene partial sequences and selected members of the *Cryptosporidium* sp. species inferred from 18S rDNA sequence comparisons. Bootstrap values (within branches at relevant nodes) are reported when equal or greater than 40%.

Table 4.26 Comparison of genotyping results for *gdh* PCR-RFLP and SEQUENCING

Isolates sequenced	Host	Origin	Genotyping results	
			GDH- RFLP	GDH- SEQ
N15408	Human	*G. lamblia* from diarrhoeal stool	Genotype/ assemblage **B**	Genotype/ assemblage **B**
GPML 1037	Human	*G. lamblia* from diarrhoeal stool	Genotype/ assemblage **B**	Genotype/ assemblage **B**
***GPML 983**	Human	*G. lamblia* from diarrhoeal stool	Genotype/ assemblage **B**	**-**
***GPML 1443**	Human	*G. lamblia* from diarrhoeal stool	Genotype/ assemblage **B**	**-**

*Isolates had short sequences, and so not included in the phylogenetic analysis

Table 4.27 Comparison of genotyping results for *18SrRNA* PCR-RFLP and SEQUENCING

Isolate	Host	Origin	Genotyping results	
			18S rRNA - RFLP	18S rRNA- SEQ
EMO346	Human	Diarrhoeal stool	*	*C. parvum*
EMO303	Human	Diarrhoeal stool	*	*
EMO312	Human	Diarrhoeal stool	*	*
EMO289	Human	Diarrhoeal stool	*	*
EMO311	Human	Diarrhoeal stool	*	*
EMO325	Human	Non-diarrhoeal stool	*	*

**Cryptosporidium* but species not very clear

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4.3 Co-infection of *G. lamblia* and rotavirus

The patients who were used for this study were children (≤ 5 years old) hospitalized for acute diarrhoea at the Children's hospital (PML, Accra). Throughout the period of hospitalization the patients received various forms of medication for treatment of diarrhoea and other clinical symptoms, and days of diarrhoea persistence observed. The diarrhoeal episodes were further categorized into acute, relapse, chronic, or recurrence (Ajjampur et al. 2009; Bilenko et al, 2004). The diarrhoea was considered to be acute if it persisted for fewer than 4 days, and persistent diarrhoea if more than 14 days. Also, patients with diarrhoea more than 3 days but fewer than 14 days were described as indeterminate, whilst a second episode commencing between 2 and 7 days after the conclusion of the original diarrhoea was described as relapse. In the present study, the maximum number of days of diarrhoea persistence was 6 days, with majority being acute cases, and there were no cases of relapse or persistent diarrhoea (Table 4.28). Apart from two patients who died on admission, all the others were treated and discharged. Management of infections by clinicians and nurses during hospitalization was generally by ORS by cup, nasogastric ORS, intravenous fluids, antibiotics, antipyretics, and antimalarials.

4.3.1 Rate of *Giardia* and rotavirus co-infections

The results obtained from the study shows that, out of a total of 194 patients who had diarrhoea, there were 22 cases with *G. lamblia* infections, that is prevalence of 11.3%, and 78 cases for rotavirus, also giving a prevalence of 40.2%. In all, 10 cases had both *G. lamblia* and rotavirus (i.e. co-infections), representing rate of co-infections of 10.3%. Unfortunately, other parasites were not tested for in this study as a result of limited funding.

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Table 4.28 Categorization of diarrhoeal cases into acute, chronic, relapse, recurrent.

Diarrhoea persistence (days)	Category of diarrhoea episode	Number of cases
1	acute	45
2	acute	50
3	acute	39
4	indeterminate	18
5	indeterminate	7
6	indeterminate	1

Table 4.29 Comparison of clinical features of children with *Giardia* and rotavirus co-infection with children with rotavirus alone.

Clinical manifestations	Episode with rotavirus alone, N= 69	Episode with rotavirus and *G. lamblia,* N=10	*P-value
1. Median duration of diarrhoea	2 (1-3)	2 (1-3)	-
2. Severity of diarrhoea (maximum number of stools/day)	2	0	0.01
< 3 episodes/day (1)	49	4	0.02
3 episodes/day (2)	13	3	0.03
4-6 episodes/day (3)	0	0	0.05
>6 episodes/day (4)			
3. Associated Fever	40	8	0.04
38-40°C (1)	27	2	0.08
> 40°C (2)			
4. Associated vomiting	5	2	0.19
1 (1)	23	3	0.24
2 (2)	34	4	0.54
3+ (3)			

*Fisher's exact test was used for all analysis, except comparison of the median duration of diarrhoea for which the Wilcoxon signed-rank test was used.

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Clinical manifestations investigated generally for all cases in this study (i.e. whether single infection or co-infections) included duration of diarrhoea, vomiting, number of times during the day and during each night, fever, and days of hospitalization were noted. The number of days of diarrhoea ranges from 1 – 5 days, with a range of 1- 6 times of stool during the day, and 1- 8 times in the night. Similarly, vomiting lasted for 1-6 days, occurring 0 -6 times during the night and 1- 6 times in the day. Temperatures measured ranges from 36°C to 40.2°C, and fever was present in 101 cases.

4.3.2 Co-infections and clinical manifestations

The severity score for diarrhoea episode (shown in Table 4.29) was computed by previously used methods (Bilenko *et al*., 2004; Ajjampur *et al*., 2009). The method assesses, number of days with diarrhoea, number of stools passed on the severest day, number of episodes of vomiting on worst day, highest temperature measured, and number of days of hospitalization. The scoring was done as follows: Days of diarrhoea: < 2 = 1 point, 2-4 days = 2 points, and >4 days = 3 points.

Number of stools per day passed: < 3 = 1 point, 3 = 2 points, 4-6 stools = 3, and > 6 stools = 4 points. Episodes of vomiting: 1 = 1 point, 2 = 2, 3+ = 3 points, and Fever: 38-40°C = 1 point, > 40°C = 2 points

4.3.3 Modulations of clinical manifestations

A critical assessment (analysis) of information obtained for children with rotavirus infection alone, compared with children with both rotavirus and *G. lamblia* (shown on Table 4.29) provides the means of determining whether *G. lamblia* was able to modulate rotaviral diarrhoea symptoms in this study. Generally, if the presence of *G. lamblia* modifies the clinical expression of infection with other pathogens, it could affect the incidence of

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diarrhoea in a given population, and its duration or its overall severity. The report from this study shows that duration of diarrhoea was the same with an average of 1-3 days, for cases with only rotavirus and also cases of co-infection with rotavirus and *G. lamblia*. With regards to severity of diarrhoea, the study also reports a significantly higher value for cases of rotavirus infection alone, as compared with cases of co-infection with *G. lamblia* which suggests modulation of infection by *G. lamblia*. However, there was no significant difference ($P > 0.05$) in vomiting for the two situations. Similarly, there was no statistically significant difference for associated fever with the groups compared. The mentioned observations reject the proposed hypothesis that *G. lamblia* could modulate clinical symptoms.

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CHAPTER FIVE

DISCUSSION

5.1 Discussion

Diarrhoeal diseases remain common among children in many developing countries including Ghana, causing considerable amount of morbidity and mortality. *Giardia lamblia* and *Cryptosporidium* sp. are medically important entero parasites which are both associated with diarrhoea especially in communities without proper sanitation and potable water.

In the present study, the prevalence rate of both parasites among children hospitalized for suffering acute diarrhoea at the Princess Marie Louis Children's hospital (PML), Accra, was studied. The reports show a 58% rate of *G. lamblia* infections and 22.2% rate of *Cryptosporidium* sp., and both infections were significantly higher than infections among the control group. The controls in this study were patients of same age who had been admitted at the hospital for illness other than diarrhoea. This is an indication that both parasites are associated with causing diarrhoea among children. In this study, there were more infections of *Cryptosporidium* sp. than *G. lamblia*, which also seems to suggest that presently in Accra, cryptosporidial diarrhoea appears to be more common than giardial diarrhoea. In a recent study involving the aetiology of paediatric diarrhoea in southern Ghana Opintan *et al.* (2010), revealed that *Cryptosporidium* infections had a prevalence of 8.7% with no *G. lamblia* present at all. Their study also took place at the Princess Marie Louise hospital, PML in Accra. The present study also appears to confirm the previous observations that cryptosporidial diarrhoea is more common than giardial diarrhoea.

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Similar findings have been made worldwide, including Saudi Arabia where *Cryptosporidium* infections among children with diarrhoea was 32% whilst *G. lamblia* was 29% (Al Braiken *et al*., 2003).*Cryptosporidium* infection was also higher than *G. lamblia* at Cuilapa Regional Hospital and five other health posts at Guatemala Nueva Santa Rosa (Velasquez *et al*., 2011). However, other studies also reported observations that were in contrast to the present study. In the Kaduna state of Nigeria, *G. lamblia* infections (3.2%) was higher than *Cryptosporidium* infections (1.9%) (Makai *et al*., 2012). Also, Eyasu *et al.* (2010) reported that among Ethiopian children, *G. lamblia* infection was 26.6% whilst *Cryptosporidium* infections was 8.1%. Although contradictory results have been obtained, the information gathered from the present study will be vital for public health authorities and policy makers who will like to embark on intervention strategies to help reduce incidence of diarrhoea in Accra. Knowing which parasite is more prevalent, will enable public health authorities be directed to where to concentrate their control efforts or activities. In this situation however, both parasites are likely to demand common source of intervention.

The occurrence of these parasites among children in selected day care centres in Accra was also investigated. Both parasites were present in all three day care centres in which the study took place, although prevalence was comparatively lower. Infections here were of asymptomatic type as none of the children had any signs of diarrhoea or other clinical symptoms. The significance of asymptomatic infections is that, those infected are not noticed to be given the appropriate medical treatment, and as they interact with other apparently healthy children they could be a source of infection, especially to those with low immunity. In order to help reduce incidence of paediatric diarrhoea in Accra, another strategy will be a regular screening and treatment of all children in day care centres. The ability to report an accurate prevalence rate depends on suitability of the laboratory methods applied for testing

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samples. To improve upon diagnosis of the parasites in stool samples, each sample was tested microscopically, and also by the enzyme immunoassay kits.

One important objective of the present study was to identify any associated risk factors for *G. lamblia* and *Cryptosporidium* infections among the children who had diarrhoea. Knowledge of associated risk factors with *G. lamblia* and *Cryptosporidium* infections will contribute towards measures that can be taken to avoid infection.

In the present study, age and sex of children were studied to ascertain whether a particular sex or age group has significantly more infections than the others. The results show that gender did not affect both *G. lamblia* and *Cryptosporidium* infections. Similar observations were made among Ugandan children who were hospitalized for diarrhoea at the Mulago hospital in Uganda (Tumwine *et al*., 2003). Also, Bello *et al*. (2011) did not find any marked difference in infection between both sexes among Cuban children. In a previous study in Cuba, Pelayo *et al.* (2008) had reported that gender did not present any differences in infection rates for the two parasites. However, a study in Ethiopia showed that more females were infected with giardiasis than males, but for cryptosporidiosis, there was no association with a particular sex group (Eyasu *et al*., 2010). A higher prevalence of giardiasis in boys than girls was also observed among Brazilian children in the region of Presidente Prudente, Sao Paulo, Brazil (Tashima *et al*., 2009). As a matter of fact, in many places worldwide, children of both sexes engage in almost the same recreational activities and so are likely to be equally exposed to any environmental contamination. Based on that assumption, observations made in the present study were expected.

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In the hospital-based study, the highest infection of *G. lamblia* occurred among children of age 2 to 3 years, and generally low for children, 1 year and below. For *Cryptosporidium,* infections occurred predominantly among age group 3 and below, with no infection among those more than 3 years. The observed pattern was different among children in the community study, that is, among children at the day care centres, as they did not follow any specific trend. In many studies *G. lamblia* infection was predominantly high in older children than in the infants (Pereira *et al.,* 2007; Escobedo *et al*., 2010; Bello *et al*., 2011; Mukherjee *et al*., 2009). Similarly, reports from investigators worldwide indicate that most infections occur among younger children of ages 2 years and below (Alexander *et al*., 2012; Wangeci *et al*., 2006; Adjei *et al.,* 2004). The observations from the present study therefore agree with many others worldwide. However, in Lagos, Nigeria, both *Cryptosporidium* and *G. lamblia* were most common among the age group 4- 5 years (Wellington *et al*., 2009), in contrast to findings of the present study.

The general notion held by many people worldwide is that women who are highly educated are likely to practice personal hygiene better than those who have only low level of education, or illiterate. Since level of sanitation and hygiene both affect transmission of these parasites, it is expected that the rate of infections among children of illiterate mothers will be higher than that of mothers with a high degree. In the present study, there was no correlation of educational background with infection rates of both parasites. The trend in observations made could be due to the influence of other factors which include the type of occupation of mother and time spent with child, and domestic support the mothers obtain from their husbands.

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Observations made in a study conducted in a semi-urban slum in India (Ajjampurr *et al*., 2010) revealed that children whose mothers had no formal education suffered multiple symptomatic *Cryptosporidium* infections significantly. Fraser *et al.* (2000) had similar findings, in which, in a birth –cohort study in Israel, it was revealed that infants whose mothers had no formal education significantly suffered from *G. lamblia* infections. Hussein (2010) observed that in Iraq, children whose mothers were complete illiterate had highest prevalence of *G. lamblia* whilst the lowest infections rate occurred among children whose mothers had completed high school or university education.

Breast milk has been highly recommended for mothers to use in feeding their infants worldwide because of its immunological and nutritional protection value. A lot of mothers do not pay much attention to it, and they continue to supplement breastfeeding with other milk products. The World Health Organization (WHO) has provided a guide for mothers to know when they can supplement infant feeding with other foods. According to the guide, mothers are advised to practice exclusive breastfeeding for at least 6 months. Exclusive breastfeeding has been reported to be more protective than the partial one. In this study, the effect of breastfeeding habits on *G. lamblia* and *Cryptosporidium* infections was investigated. Majority of mothers (218) practiced exclusive breastfeeding whilst, a minority (131) did not. The results show that breastfeeding habits did not have any influence on the rate of infection of both parasites in the children. This observation is in agreement with earlier report from investigations made among Brazilian children hospitalized for diarrhoea (Pereira *et al.,* 2007). In contrast to observations made in the present study, Bilenko *et al.* (2008) reported from a birth cohort study, that children in Israel who were exclusively breastfed received at least, some protection from *Cryptosporidium* sp.and *G. lamblia* infections within the first 3 months after birth. Other studies support the ability of exclusive breastfeeding to reduce

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infections of the two parasites among children (Pelayo *et al*., 2008; Mahmud *et al*., 2001). In the present study, other factors such as source of food and water might have contributed to some infections that occurred in children who had been breastfed exclusively. The subject of exclusive breastfeeding in relation to enteric parasitic infections in Accra needs further investigation.

With regards to source of drinking water for the children, three main categories were identified. These were children who depended on pipe-borne water only, sachet (bagged) water only, and those who depended on both. There were much more infections of both parasites associated with drinking of sachet water. This observation made was expected, as the purity and safety of sachet water in Accra has been already challenged by some investigators. After screening an appreciable number of sachet water of different brands, obtained from vendors on the street of Accra, Kwakye-Nuako *et al.* (2007) reported that seventy-seven per cent (77%) of the samples contained parasitic agents including oocysts of *Cryptosporidium* sp.. Generally, the treatment of water to eliminate any infestation by *G. lamblia* and *Cryptosporidium* has not been completely successful. The cyst and oocyst of the parasites have not only been able to escape the filtration process due to their extremely small size, but have also been resistant and unaffected to chemicals used for treatment. To ensure public health safety of the consumption of sachet water in Accra, there is the need for regular screening and monitoring of sachet water sold in the city of Accra.

In Cuba, Bello *et al*. (2011) observed that children from households that did not receive water from an aqueduct were at relatively high risk of *Giardia* infection as were those who drank unboiled tap water. In another study at Northwestern Ethiopia, it was observed that the source of water supply to a community could be a source of *Giardia* or *Cryptosporidium* infection

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(Eyasu *et al.,* 2010). The manner in which food is handled by people, especially among food vendors is very important as it could introduce infection of one pathogen or the other to consumers. To ensure public health safety, maximum hygienic practice should be strictly observed by all food vendors on the street. In the present study, the source of food for the children, and its relation to infection of any of the parasites was investigated. Infections in children who obtain their food from the street vendors were compared with infections in those whose food are always prepared at home. The results show that children whose food was prepared at home were not necessarily protected from parasitic infection. It could be that the hygienic handling of food is not being practiced as expected, or other factors influenced the detection rate that was observed in the present study.

The keeping of pets and rearing of domestic animals at home is a common practice in the Ghanaian society. In this study, a higher percentage of children had animals at home, and these were either dogs or cats, or both. Usually these animals defaecate on the compound soil, and sometimes dry up on the soil to contaminate the environment, which also serves as playing grounds for children. Being zoonotic infections, it is suspected that some level of infections of the parasites could come from infected animals kept at home. Reports from the study indicate, on the contrary that, having one or more pets or domestic animals at home was not a risk factor for acquiring *G. lamblia* or *Cryptosporidium* infections. Perhaps, owners of such pets or domestic animals observed good hygiene and sanitation in their homes to prevent infection. A direct and better assessment however will have been a comparison of genotypes detected from the stool of children with genotypes also detected from the stools of domestic animals. There are reports worldwide on associated risk of *G. lamblia* and *Cryptosporidium* infections in people who keep domestic animals at home (Pinheiro *et al.*, 2011; Ratanapo *et al.*, 2008; Pereira *et al.*, 2007).

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The rate of infection of the parasites was also studied across dry and wet seasons. The observation made was that, although *G. lamblia* and *Cryptosporidium* were present all year round, seasonal variations were not significant. The pattern appears to be in line with availability of water. In many communities in Accra, there is inadequate supply of treated drinking water to homes of many people all year round, and as they depend on other sources for water, the risk of infections also remains all year round.

In other places, more infections are reported during the hot season than the cold season (Wangeci *et al*., 2006; Wongstitwilairoong *et al*., 2007; Hussein, 2010), and the investigators of those studies explain that people consume more water in the hot season which tends to increase the chances of getting infected. Many investigators have also reported the occurrence of more infections during the rainy (wet) season than the dry season (Alexander *et al*., 2012; Siwila *et al*., 2011). According to the authors, this is the period that a lot of water bodies get contaminated with faecal matter deposited on soils, which might contain cysts or oocysts of the parasites, through runoffs. It tends to increase the risk of infections among people who depend on such water bodies.

The recent application of molecular tools in medical research has enabled researchers to identify sources of infection of some diseases. Giardiasis and Cryptosporidiosis are both zoonotic, and in many countries where the diseases are common, researchers have tried to determine the zoonotic pathway of transmission. In this study, isolates of the parasites detected were genotyped through PCR-RFLP and nucleotide sequence analyses successfully. Giardia genotype (assemblage) B was observed to be the only genotype identified in both diarrhoeic and non-diarrhoeic children. The significance of this finding is that, children in Accra are getting infected through, human-to-human transmission, as the genotype B has

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been reported to be exclusively anthroponotic (Abe *et al.,* 2005; Caccio *et al*., 2005; McLauchlin *et al.,* 2000). Abe and Teramoto (2011) made similar reports when they observed that genotype B was the only genotype detected among patients and health care workers at Osaka city, Japan. Among Saudi children, all assemblage B detected was associated with diarrhoea (Al-Mohammed, 2011). Although genotypes A and B were identified among Egyptian children, genotype B was more associated with diarrhoea than genotype A (Foronda *et al*., 2008). Similar observations were made among Indian children (Ajjampur *et al*., 2009), Ethiopian children (Gelanew *et al*., 2007) and more recently in Guinea Bissau (Fereira *et al*., 2012), all showing predominance of genotype B in diarrhoeal cases.

Also, in this study, the *Cryptosporidium parvum* species was identified as one associated with diarrhoea among children in Accra. The other *Cryptosporidium* sp. other than '*parvum',* also detected in the present study could not clearly be identified, as none of them belonged to *hominis*, *andersoni, muris, gali*, or any other as revealed through the phylogenetic analysis. Those species are suggestive of new species emerging in the population of Accra, and further investigations are needed to confirm their identity as truly new emerging species..

Cryptosporidium parvum is generally involved in both zoonotic and anthroponotic transmissions. It comprises of Subtype 1 which is anthroponotic and subtype 2 which is zoonotic. In this study sub-genotyping analysis was not performed, making it difficult to specifically report whether animals were involved in transmission. Identifying just one type of genotype for *Giardia* (genotype B) and one species of *Cryptosporidium* sp. (i.e. *parvum* only) suggests that in Accra, Ghana, *G. lamblia* is not highly diversified, as among other populations elsewhere. Since sub-genotyping analysis was not performed for

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Cryptosporidium parvum, it will not be possible to completely describe diversity of *Cryptosporidium* sp. in Accra. In Nigeria, Molloy *et al.* (2010) identified *C. hominis, C. parvum, C. meleagridis* and a few others in the population studied. Also, among Peruvian children Cama *et al.* (2008) reported that *C. hominis, C. parvum, C. meleagridis* were all present together with subtypes Ia, Ib, Id and Ie.

In studying the co-infections of rotavirus and *G. lamblia,* and whether the clinical symptoms are modulated or made less severe in co-infections compared with only rotaviral diarrhoea, the observations made did not allow a conclusive remark. There were a number of limitations in the study which include my inability to screen other parasites or enteropathogens that are also associted with diarrhoea. Secondly, there will be the need to compare prevalence of both cases, that is, rotavirus alone with rotavirus and *G. lamblia* with other enteropathogens for a period, as reported by Ajjampur *et al.* (2009). In a community birth-cohort study, high prevalence rate of asymptomatic Giardia- rotavirus co-infection cases could mean modulation of clinical symptoms of rotaviral diarrhoea. Thus, further investigations are needed on the subject of co-infection with *G. lamblia* to confirm the observations by Bilenko *et al.* (2004), who suggested that there was a modulating effect of clinical symptoms of rotaviral diarrhoea by presence of *G. lamblia* among the Bedoiun children in Israel.

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5.2 CONCLUSION, LIMITATIONS AND RECOMMENDATIONS

Information on the application of molecular techniques in medical research in Ghana continues to improve by way of more studies being conducted recently, mostly, with collaboration from researchers with advanced laboratory facilities abroad. In many countries throughout the world where giardiasis and cryptosporidiosis are common, efforts have been made to scientifically determine the source of infection. The recent work is an addition which addresses the issue of sources of contamination in our environment at Accra,that can lead to *G. lamblia* and *Cryptosporidium* sp. infections. The molecular characterization study in the present work has revealed that genotype B of *G. lamblia* which happens to be strictly anthroponotic is the only genotype infecting children in Accra. Since the involvement of animals in the transmission of this parasite was not established in this study, people with pets and other domestic animals in their homes do not have any risk of *G. lamblia* infections from keeping them.

In the case of *Cryptosporidium,* the 'parvum' species together with others with unclear identity were observed. *C. parvum* has subtypes, namely, genotype 1 and genotype 2. Sub-genotyping techniques are needed to find out which type of *C. parvum* exist among children in Accra, and subsequently be able to determine whether they are zoonotic or anthroponotic species or both. The hospital-based study indicates that currently, *Cryptosporidium* parasite is more associated with paediatric diarrhoea than *G. lamblia*. This observation suggests that much more attention be given to cryptosporidial diarrhoeal, especially source of infection.

The mode of infection is through the intake of water contaminated with oocysts of the parasite. This study identified that sachet (bagged) water was one of the main sources of

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drinking water to the children studied at the hospital. Drinking sachet water had the highest odds (1.935) for risk of *Cryptosporidium* infections, and 1.645 odds of *G. lamblia* infections. The issue about sachet water needs an urgent attention by public health authorities, since an earlier report in a study at Accra indicates that some sachet water samples collected and screened, showed a gross contamination by enteropathogenic organisms including *Cryptosporidium* oocysts.

In this study, sex of child was not associated with either infections. With regards to age, there were higher odds of risk for older children for *Giardia* infections, whilst in *Cryptosporidium* infections the highest odd was for children one year and below. Thus, there was some association with age and infections of each parasite. The effect of breastfeeding habits on infection needs further investigation, as observation from this study does not agree with many reports worldwide. Breast milk is supposed to be protective, especially if mother practices exclusive breastfeeding. The formal education level of mother was not identified to be associated with infections of any of the parasites, and therefore being illiterate was not a risk factor.

Due to the presence of these parasites among children in daycare centres in Accra, occurring as asymptomatic infections, as reported from the study, there will be the need to have frequent screening and treatment of children not only in day care centres but all other social institutions such as orphanages in Ghana. There was no seasonal variation of infections of the parasites, as they were detected all year round, that is, in rainy season and dry season,. With regards to co-infection and modulation of clinical manifestations, there were considerable limitations including difficulties in testing for the presence of other parasites and enteropathogens.

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Limitations of Study

1. Single stool collection, which might lead to under detection of parasites
2. Inability to test for other parasites which occurred together with *G. lamblia* and *Cryptosporidium*
3. As a result of limited funds, *C. parvum* isolates could not be sub-genotyped in the present study into the two main sub-groups namely, genotype 1 or genotype 2.

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Recommendations

A wider study to cover several other social institutions such as orphanages will provide more information on asymptomatic infections including prevalence and type of genotypes and species of *G. lamblia* and *Cryptosporidium* respectively.

The study concerning co-infections of rotavirus and *G. lamblia* in relation to modulation of clinical manifestation also demands further investigations, and, a community birth cohort study is recommended.

The use of single specimen collection to detect prevalence can be compared with other studies in which more than a single collection is done for each patient.

The public health risk associated with drinking sachet water needs frequent investigation. Also, there is a need for further studies on breastfeeding habits and its ability to protect children from parasitic infections.

Further studies on sub-genotyping of *C. parvum,* to determine zoonotic and anthroponotic transmissions in Accra is required.

Finally, serving as a baseline data on molecular characterization of *G. lamblia* and *Cryptosporidium* sp. the present study should be repeated from time to time, to monitor the diversity of genotypes or species and sub-species in Accra.

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APPENDIX 1

Questionnaire For the Study of

Epidemiology and Molecular Characterization of Giardiasis and cryptosporidiosis in Accra

Dear Respondent,

This questionnaire is designed for a research work from the Department of Microbiology, University of Ghana Medical School, Korle Bu. Please FILL IN correct information. All information will be treated confidentially and the information will be used for this research work only. Thank you.

Please tick [√] or fill where appropriate.

Basic Data

1. Child's
   Name……………………………………………………………Age…………..
   Weight ……………………………………………….
   Height……………………………….
2. Sex A. Male [ ] B. Female [ ] Sample No…….

Socio-economic Factors

3. Mother/Guardian's Educational qualification. A. BECE [ ] B. SSSCE [ ] C.HND [ ] D.Bsc/BA [ ] E.Msc/PhD [ ] E. others Specify…………….
4. Mother/Guardian's Occupation? A. Farming [ ] B. Fishing [ ] C. Trading [ ] D. Office work [ ] E. Others [Specify]…………………………………….

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5. Father/Guardian's Educational qualification. A. BECE [ ] B. SSSCE [ ] C.HND [ ] D.Bsc/BA [ ] E.Msc/PhD [ ] E. others Specify…………….
6. Father/Guardian's Occupation? A. Farming [ ] B. Fishing [ ] C. Trading [ ] D. Office work [ ] E. Others [Specify]…………………………………….
7. Source of drinking water. A. Pipe borne water [ ] B. Borehole [ ] C. Well [ ] D. Rain water[ ] E. Sachet water [ ].

Nutrition of Child

8. Breastfeeding Habits (children < 2 years)? A. Exclusive for 6 months [ ] B. Not Exclusive [ ] C. Not Breastfed [ ] D. Others [ ] (Specify)……………………………..
9. Source of food? A. Cooked at home only [ ] B, Bought on Street [ ] C. Both, cooked at home and sometimes obtained from street [ ] D. Others [ ] Specify ………………..

Presence of Domestic Animals

10. Presence of animals at home? Yes [ ] No [ ]

Specify type of animal A. Dog [ ] B. Cat [ ] C. Goat [ ] D. Sheep [ ] E. Fowl [ ] Others (specify)………………………………

Clinical Information /Gastrointestinal Symptoms (To be completed by Nurse/Doctor)

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11. Does child (patient) have any of the following conditions? A. Vomiting [ ] B. Abdominal pain [ ] C. General Malaise [ ] D. Fever[ ] E. Others [ ] (Specify…………………………………………………………………………………

Specify Any treatment given

……………………………………………………………….

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APPENDIX 2

SAMPLE OF INFORMED CONSENT

Title: Epidemiology and Molecular Characterization of Giardiasis and cryptosporidiosis in Accra

Principal Investigator: Isaac Anim-Baidoo

Address: Department of Medical Laboratory Science, University of Ghana School of Allied Health Sciences, P. O. Box, KB 143, Korle Bu, Accra

1. You -----------------------------------have been asked to give permission for your child ------------------------------to voluntarily participate in a research study entitled 'Epidemiology, Molecular and Immunological Characterization of Giardiasis in Ghana'. The purpose of this study is to try to find out what makes your child sick with diarrhoea. Your child's participation will be for a few minutes. During your child's participation you will be asked to answer questions regarding your child's condition, and your child will be required to give both stool and blood sample. If your child can not provide the stool sample, a swab from the rectum will be performed using a cotton bud on the tip of a small stick. The stool and blood will be used to perform laboratory tests to determine what makes your child have diarrhoea sometimes.

2. The risks of discomfort to you or your child are small, and may involve mild discomfort in providing the stool/blood sample.

3. The benefits that you may expect from you or your child's participation in this research is to know what may be causing the diarrhoea in a timely manner. There are no other benefits, costs, or compensation for you or your child expected from participation in the study. But the information gained from the study will help investigate the causes of diarrhoea circulating in the country and may help the health officials plan a strategy to combat it.

4. Laboratory results and the information regarding you or your child will be strictly confidential. Your confidentiality during the study will be ensured by using a research identification number.

5. A sample of your child's stool/blood may be stored for future laboratory diagnosis to include causes of diarrhoea due to germs. In addition, any germ that is found will be stored for future studies to the germ.

6. If you have any questions about the study, you should contact Mr. Isaac Anim-Baidoo on phone number, 233-24998702.

7. Your child's participation in this study is completely voluntary. If you do not want to participate there will be no penalty and you or your child will not lose any benefits to

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which you or your child is otherwise entitled. If you want to discontinue participation in the study at any time, there will be no penalty and you or your child will not lose any medical care or benefits to which you or your child is otherwise entitled.

8. All answers provided by the researcher are understandable to me and are satisfactory. I understand to the best of my ability what has been explained in this consent form about my child's participation in the study. I have had enough time to consider the decision to participate or have my child participate in this study and I don't need further information to make my decision, whether or not to let my child participate in the study. With my signature or thumbprint below, I give my voluntary informed consent to have my child participate in the research as it has been explained to me.

-- --------------------------
-----Signature/Thumbprint of parent/guardian Date
(DD/MM/YY)

--- ------------------------------------ -------------------------
---
Printed name of participant Printed name of parent/guardian Date
(DD/MM/YY)

9. We certify that this form was signed by the person above

----------------------------------- ------------------------------------- ------------------------
---
Printed name of investigator Signature of investigator Date
(DD/MM/YY)

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APPENDIX 3

QUESTIONNAIRE

Rotavirus Surveillance Study Hospitalization data

Eligibility: Age < 6 months +3 or more loose/watery stools per day for < 7days

BASIC DATA

1.Form serial number	[ ] [ ] [ ] [ ]	FORMNUM
2.Study ID no. (Sample no.)	[ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ]	SCID
3.Hospital folder no.	[ ] [ ] [ ] [ ] [ ] [ ] [ ]	FOLDNUM
4.Name of child………………………………………		SCNAM
5.Date of birth	[ ] [ ] [ ] [ ] [ ] [ ]	DOB
6.Age in months	[ ] [ ]	AGEMO
7.Sex	[ ]	SEX
8.Place of residence		RESNAM

Clinical data

1.Date of admission	[ ] [ ] [ ] [ ] [ ] [ ]	DATEA
2.Date of recruitment	[ ] [ ] [ ] [ ] [ ] [ ]	DATER

Please describe your child's illness (DO NOT PROMPT)

3.Diarrhoea	(Yes) (No)	DIARRHOEA
4.Vomiting	(Yes) (No)	VOMIT
5.Fever	(Yes) (No)	FEVER

(Specify admission temperature in all --------^{o}C)

6. Other (specify) -- OTHSYM

Duration of symptoms before admission

1.Date onset of diarrhoea	[ ] [ ] [ ] [ ] [ ] [ ]	DATEOD
2.Duration of diarrhoea (days)	[ ] [ ]	DIARDUR
3.Duration of vomiting (days)	[ ] [ ]	VOMDUR

Severity of symptoms at admission

When diarrhoea was at it's worst

1.How many stools during the day	[ ] [ ]	DIARDNUM
2.How many stools during the night	[ ] [ ]	DIARNUM

When vomiting was at it's worst

1.How many times during the day	[ ] [ ]	VOMDNUM
2.How many times during the night	[ ] [ ]	VOMNNUM

Severity of symptoms by Physicians clinical assessment on admission

Circle as appropriate: Sunken eyes lethargic sunken anterior frontanelle impaired consciousness unconscious

Circle as appropriate the physicians diagnosis at first assessment: [1] Mild dehydration [2]Moderate dehydration [3] Severe dehydration

Management during Hospitalization

1.ORS by cup	(Yes) (No)	MGXCUP
2.Nasogastric ORS	(Yes) (No)	MGXNG
3.Intravenous fluids	(Yes) (No)	MGXIV
4.Other medications prescribed	(Yes) (No)	OTHER1
Antibiotics	(Yes) (No)	ANTIBIOT

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Antipyretics (Yes) (No) ANTIPYR
Antimalarials (Yes) (No) ANTIMAL
Other (specify) (Yes) (No) OTHER2

Outcome and duration of hospitalization

1.Discharged (Date of discharge [ ] [ ] [ ] [ ] [ ] [ ] OUTCDS
2.Died (Date of death) [ ] [ ] [ ] [ ] [ ] [ ] OUTCDD
3.Duration of hospital admission (in hours) [ ] [ ] [ ] [ ] DURHOSP

Laboratory data

1.Date stool collection [ ] [ ] [ ] [ ] [ ] [ ] DATESC
2.Date stool sent to the laboratory [ ] [ ] [ ] [ ] [ ] [ ] DATESL
3.Date laboratory result [ ] [ ] [ ] [ ] [ ] [ ] DATELR
4.Rotavirus EIA result --- ROTAR
5.Adequacy of specimen (Yes) (No) SPECADEQ
6.Date stool sent to reference laboratory [ ] [ ] [ ] [ ] [ ] [ ] DATESRLN
7.VP7 (G) strain.. GSTRAIN
8.VP4 (P) genotype.. PTYPE

SIGN...DATE...
NAME...

COMMENTS

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APPENDIX 4

Isolates used for the molecular analysis of *G. lamblia*

Serial number	Isolate code	Origin	Microscopy	ELISA
1.	*EM0004	PML, Diarrhoea	+ (Positive)	+ (Positive)
2.	*EM0025	PML, Diarrhoea	+	+
3.	EM0027	PML, Diarrhoea	- (Negative)	+
4.	EM0029	PML, Diarrhoea	-	+
5.	EM0038	PML, Diarrhoea	-	+
6.	*EM0039	PML, Diarrhoea	+	+
7.	EM0050	PML, Diarrhoea	-	+
8.	EM0052	PML, Diarrhoea	+	+
9.	EM0078	PML, Diarrhoea	+	+
10.	EM0089	PML, Diarrhoea	+	+
11.	EM0097	PML, Diarrhoea	-	+
12.	*EM0156	PML, Diarrhoea	-	+
13.	*EM0170	PML, Diarrhoea	-	+
14.	*EM0264	PML, Diarrhoea	+	+
15.	*EM0275	PML, Diarrhoea	+	+
16.	EM0290	PML, Diarrhoea	-	+
17.	EM0316	PML, Diarrhoea	+	+
18.	EM0392	PML, Diarrhoea	+	+
19.	EM0436	PML, Diarrhoea	+	+
20.	EM0459	PML, Diarrhoea	+	+
21.	EM0462	PML, Diarrhoea	+	+
22.	EC0029	PML, Non-diarrhoeal	-	+
23.	EC0048	PML, Non-diarrhoeal	-	+
24.	EC0070	PML, Non-diarrhoeal	-	+
25.	EC0081	PML, Non-diarrhoeal	+	+
26.	EC0093	PML, Non-diarrhoeal	+	+
27.	EC0108	PML, Non-diarrhoeal	+	+

*Co-infection of *G. lamblia* and *Cryptosporidium* sp. in stool sample

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Isolates used for the molecular analysis of *Cryptosporidium* sp.

Serial number	Isolate code	Origin	Microscopy	ELISA
1.	EM0002	PML, Diarrhoea	+(Positive)	+ (Positive)
2.	*EM0004	PML, Diarrhoea	+	+
3.	*EM0025	PML, Diarrhoea	- (Negative)	+
4.	*EM0039	PML, Diarrhoea	-	+
5.	EM0051	PML, Diarrhoea	-	+
6.	EM0065	PML, Diarrhoea	-	+
7.	EM0071	PML, Diarrhoea	-	+
8.	EM0073	PML, Diarrhoea	-	+
9.	EM0090	PML, Diarrhoea	-	+
10.	EM0105	PML, Diarrhoea	-	+
11.	EM0110	PML, Diarrhoea	-	+
12.	EM0119	PML, Diarrhoea	-	+
13.	EM0122	PML, Diarrhoea	-	+
14.	EM0125	PML, Diarrhoea	+	+
15.	EM0127	PML, Diarrhoea	-	+
16.	EM0129	PML, Diarrhoea	-	+
17.	EM0133	PML, Diarrhoea	-	+
18.	EM0137	PML, Diarrhoea	-	+
19.	EM0140	PML, Diarrhoea	-	+
20.	EM0143	PML, Diarrhoea	+	+
21.	EM0152	PML, Diarrhoea	-	+
22.	EM0154	PML, Diarrhoea	+	+
23.	*EM0156	PML, Diarrhoea	-	+
24.	EM0159	PML, Diarrhoea	-	+
25.	EM0162	PML, Diarrhoea	+	+
26.	EM0163	PML, Diarrhoea	-	+
27.	EM0164	PML, Diarrhoea	-	+
28.	EM0165	PML, Diarrhoea	-	+
29.	EM0166	PML, Diarrhoea	+	+
30.	EM0169	PML, Diarrhoea	+	+
31.	*EM0170	PML, Diarrhoea	-	+
32.	EM0173	PML, Diarrhoea	-	+
33.	EM0175	PML, Diarrhoea	-	+
34.	EM0186	PML, Diarrhoea	+	+
35.	EM0190	PML, Diarrhoea	+	+
36.	EM0192	PML, Diarrhoea	+	+
37.	EM0193	PML, Diarrhoea	-	+
38.	EM0199	PML, Diarrhoea	-	+
39.	EM0204	PML, Diarrhoea	-	+
40.	EM0206	PML, Diarrhoea	+	+
41.	EM0212	PML, Diarrhoea	-	+
42.	EM0263	PML, Diarrhoea	+	+
43.	*EM0264	PML, Diarrhoea	-	+
44.	*EM0275	PML, Diarrhoea	+	+
45.	EM0288	PML, Diarrhoea	+	+
46.	EM0292	PML, Diarrhoea	+	+

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47.	EM0294	PML, Diarrhoea	+	+
48.	EM0296	PML, Diarrhoea	-	+
49.	EM0297	PML, Diarrhoea	-	+
50.	EM0300	PML, Diarrhoea	+	+
51.	EM0304	PML, Diarrhoea	-	+
52.	EM0305	PML, Diarrhoea	-	+
53.	EM0306	PML, Diarrhoea	+	+
54.	EM0310	PML, Diarrhoea	+	+
55.	EM0320	PML, Diarrhoea	-	+
56.	EM0321	PML, Diarrhoea	-	+
57.	EM0323	PML, Diarrhoea	-	+
58.	EM0325	PML, Diarrhoea	-	+
59.	EM0329	PML, Diarrhoea	-	+
60.	EM0332	PML, Diarrhoea	+	+
61.	EM0333	PML, Diarrhoea	-	+
62.	EM0341	PML, Diarrhoea	+	+
63.	EM0345	PML, Diarrhoea	+	+
64.	EM0353	PML, Diarrhoea	+	+
65.	EM0355	PML, Diarrhoea	-	+
66.	EM0356	PML, Diarrhoea	-	+
67.	EM0359	PML, Diarrhoea	-	+
68.	EM0363	PML, Diarrhoea	-	+
69.	EM0373	PML, Diarrhoea	-	+
70.	EM0389	PML, Diarrhoea	+	+
71.	EM0405	PML, Diarrhoea	-	+
72.	EM0419	PML, Diarrhoea	+	+
73.	EM0420	PML, Diarrhoea	+	+
74.	EM0423	PML, Diarrhoea	-	+
75.	EM0424	PML, Diarrhoea	-	+
76.	EM0432	PML, Diarrhoea	-	+
77.	EM0435	PML, Diarrhoea	+	+
78.	EM0438	PML, Diarrhoea	+	+
79.	EM0458	PML, Diarrhoea	-	+
80.	EM0471	PML, Diarrhoea	-	+
81.	EM0486	PML, Diarrhoea	-	+
82.	EC0008	PML, non-diarrhoeal	-	+
83.	EC0024	PML, non-diarrhoeal	+	+
84.	EC0073	PML, non-diarrhoeal	+	+
85.	EC0090	PML, non-diarrhoeal	-	+

*Co-infection of *G. lamblia* and *Cryptosporidium* sp. in stool sample

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APPENDIX 5

<u>Nucleotide sequence analysis of *Cryptosporidium* sp.</u>

```
>muris
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tcctattatattctaaatatataggaaattttactttgagaaaattagagtgcttaaagcaggcaactgcc
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cctta

>parvum
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tta
```

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>parvum strain H
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tcagataccgtcgtagtcttaaccataaactatgccaactagagattggaggttgttccttactccttcag
caccta

>parvum Strain C bovine
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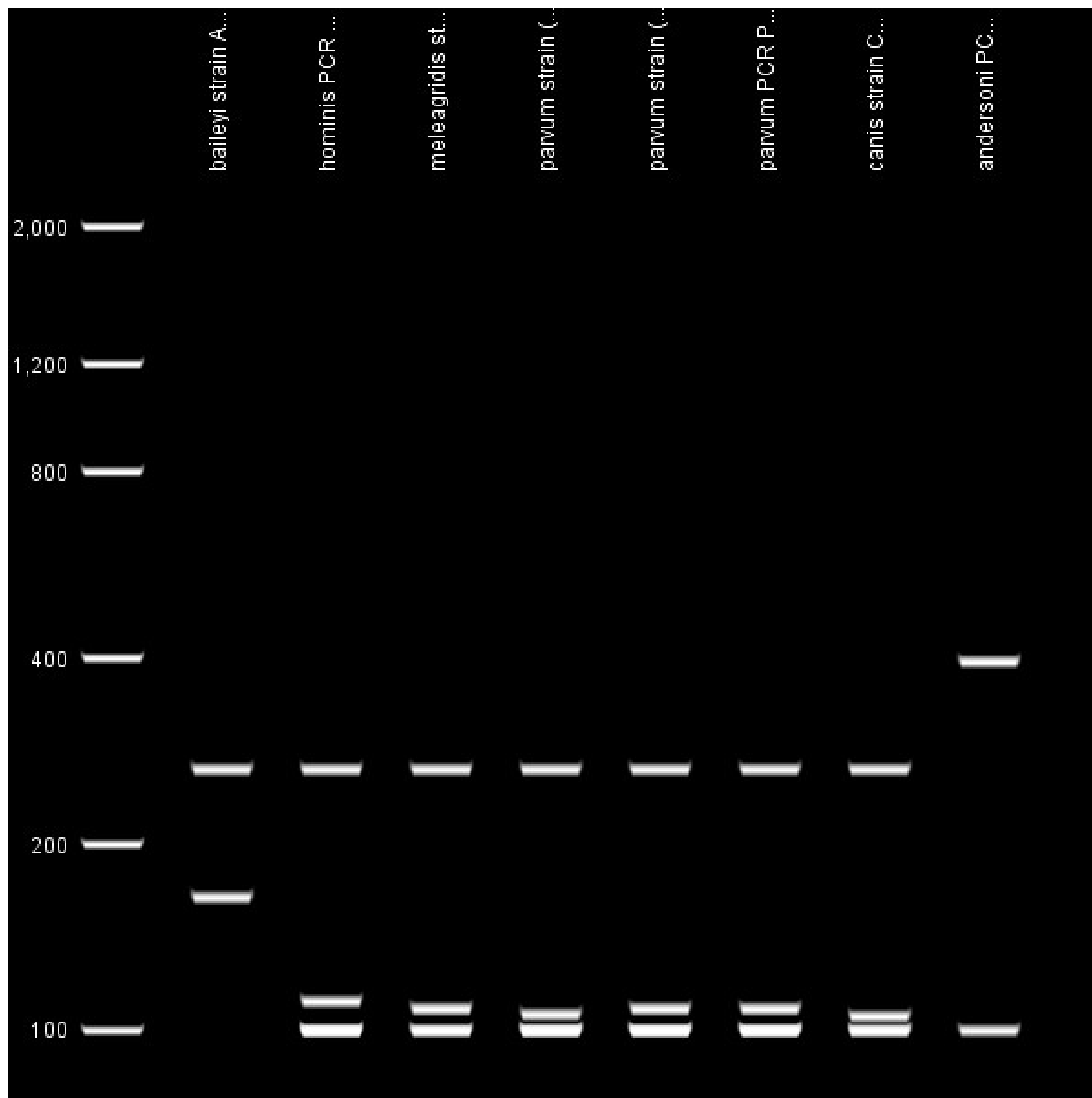
APPENDIX 6



Gel electrophoresis picture of digestion of 18S rRNA *Cryptosporidium* sp. by SspI restriction enzyme (*in-silico analysis*)

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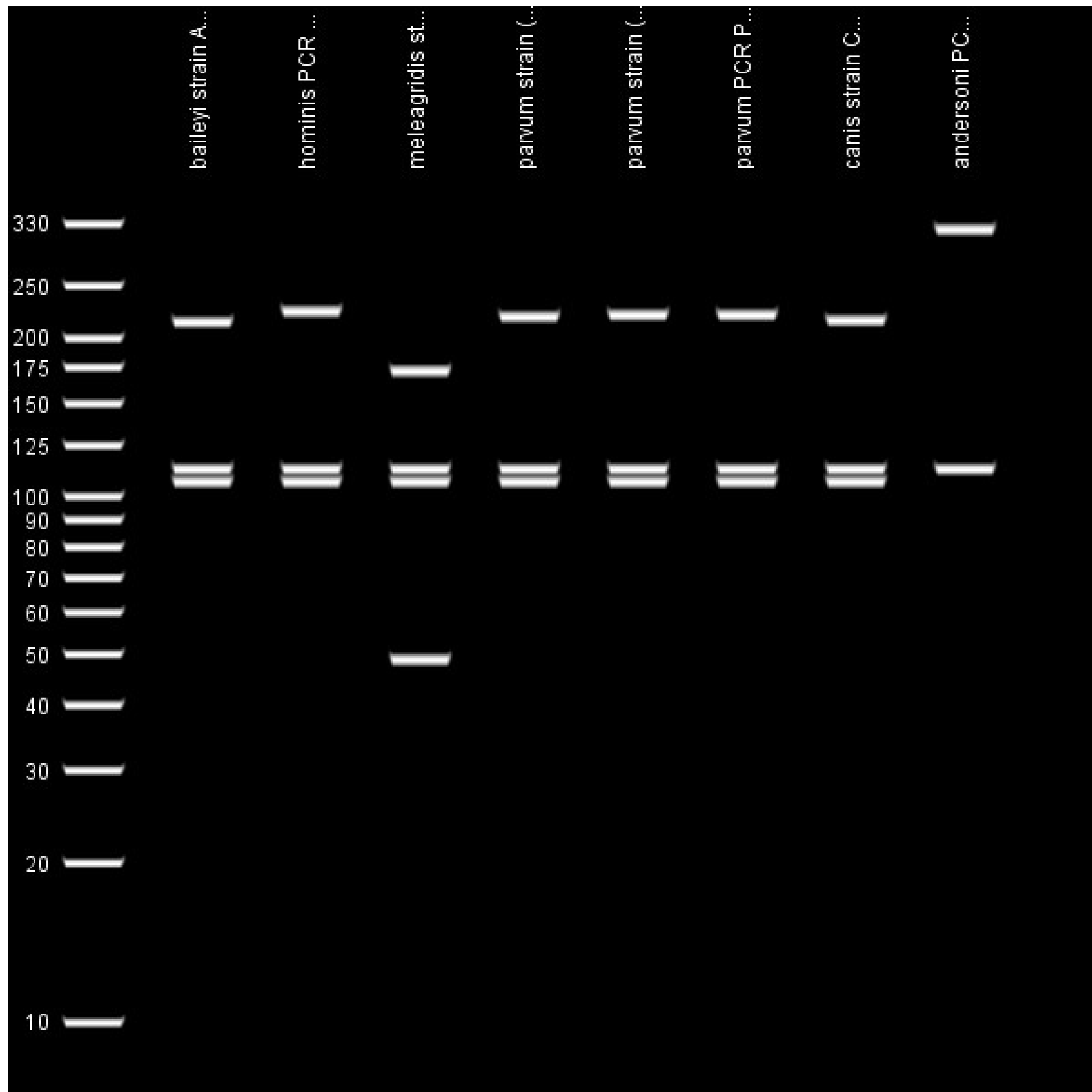
APPENDIX 7



Gel electrophoresis picture of digestion of 18S rRNA *Cryptosporidium* sp. by VspI restriction enzyme (*in-silico analysis*)