THE MOLECULAR CHARACTERISATION OF GIARDIA LAMBLIA IN GHANAIAN CHILDREN

BY

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JULY, 2013
DECLARATION

I hereby declare that except for references to other people’s work, which have duly been acknowledged, this exercise is a result of my own research and this thesis neither in whole nor in part, has been presented for another degree elsewhere.

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DEDICATION

To the Oddei family, Emmanuel my dad, Comfort my mum, my siblings Jones, Joseph, Fred, Emmanuel, David and to my sweetheart Louis Obeng Darko. I just would not have had anyone to replace each of you in my life. I appreciate and love you all. God richly bless you.
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Diarrhoea is among the six major diseases that causes death among children below age five worldwide. It is also a major cause of morbidity and mortality among children below age five in Ghana. *Giardia lamblia* is among the major enteropathogen causes of diarrhoea in Ghana. Most studies in Ghana on detecting *G. lamblia* use microscopy and enzyme linked immunosorbent assay. There is not much data on the molecular epidemiology of Giardiasis in Ghana. This study was conducted to determine the prevalence and genotype(s) of *Giardia lamblia* among children up to five years of age with diarrhoea at the Princess Marie Louise Hospital. Stool samples were collected and transported on ice to the Parasitology Department of the Noguchi Memorial Institute for Medical Research for analysis. In the laboratory, the presence or absence of *G. lamblia* and its genotype was analyzed using a nested polymerase chain reaction, restriction fragment length polymerase chain reaction and DNA sequencing. Demographic and clinical data was also obtained from the hospital. The associations between demographic, clinical and genotype present was assessed by $\chi^2$ significance set at <0.05. The prevalence of *G. lamblia* from the stool samples was 6.2%. Only genotype B was detected through RFLP. The genotype was also confirmed by DNA sequencing. There was no significant association between clinical symptoms and the genotype recorded but it could be observed the genotype of *G. lamblia* is more prevalence among people with symptomatic giardiasis.
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<tr>
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<th>Full Form</th>
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<tbody>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Disodium ethylene diamine tetraacetate</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium Bromide</td>
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<tr>
<td>H₂O</td>
<td>Water</td>
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<tr>
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<td>Molar</td>
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<tr>
<td>mg</td>
<td>Milligram</td>
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<tr>
<td>ml</td>
<td>Millitre</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>pH</td>
<td>Hydrogen-ion exponent</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolution per minute</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>sddH₂O</td>
<td>Sterile double distilled water</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-actate EDTA</td>
</tr>
<tr>
<td>Tris</td>
<td>2-amino-2-hydroxymethyl-1,3-propanediol</td>
</tr>
<tr>
<td>µ</td>
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</tr>
<tr>
<td>µg</td>
<td>Micro gram</td>
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<td>Micro litre</td>
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<td>µM</td>
<td>micromolar</td>
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CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 Introduction

One of the main aims of the millennium development goal four is to reduce by two-thirds the mortality among children below the age five by 2015 (Bryce et al., 2005). Of the 10.6 million recorded deaths in children less than age five, 73% are attributed to six major health related causes. These are pneumonia, diarrhoea, malaria, sepsis, preterm delivery and asphyxia. Amongst these, diarrhoea alone accounts for 18% and is the second major cause of death (Bryce et al., 2005). Diarrhoea diseases kill about 750,000 children in a year (WHO, 2012). The median of episodes of diarrhoea per year in developing countries for children aged less than 59 months is 3.2 and the estimated mortality is 4.9 per 1000 per year. According to Kosek et al., (2003), 21% of children in developing countries die before age of five as a result of diarrhoea.

Diarrhoea is also a major cause of mortality and morbidity among children below the age of five years in Ghana. This is attributed mainly to the consumption of contaminated water and the unhealthy practices in food preparation and disposal of excreta (The Ghana Demographic and Health Survey, 2009). Most of the intestinal parasites present in the diarrhoeal stools of children in a hospital-based study in Ghana were Giardia lamblia, Trichomonas hominis, Trichuris trichuria and Hookworm (Addy et al., 2004).

Diarrhoea is defined as the passage of three or more loose or liquid stools per day or more frequently than is normal for an individual (WHO, 2012). Infections that result in diarrhoea are transmitted through contaminated food, drinking water or from person to person as a result of poor hygiene. It is usually a symptom of infections of the intestinal tract which may be caused by a variety of infectious agents including bacteria, virus and
parasitic organisms. Diarrhoea is the major symptom associated with symptomatic giardiasis (Heresi and Cleary, 1997).

Giardiasis is a diarrhoeal illness caused by the parasitic protozoan flagellate, *G. lamblia* (Kappus *et al.*, 1994). About 200 million people have symptomatic giardiasis in Asia, Africa and Latin America, with 500,000 new cases reported each year (WHO, 2011). *Giardia lamblia* is one of the enteroproteozons of significance to man (Norhayati *et al.*, 2003). The disease has been found in almost all forms of vertebtrates including humans, domestic animals, wildlife and marine vertebtrates, giving it a zoonotic importance (Lasek-Nesselquist *et al.*, 2008).

*Giardia lamblia* isolates from humans has been genotyped into assemblages A and B, which have been further genotyped into sub-assemblages AI, AII, BIII and BIV (Monis *et al.*, 1996; Monis *et al.*, 2006). Isolates A and B have also been detected in other vertebtrates including dogs, cats, livestock and beavers (Monis *et al.*, 1999). The various genes of importance for genotyping *G. lamblia* include the small sub unit ribosomal DNA, variable surface protein, glutamate dehydrogenase, triosephosphate isomerase, elongation factor alpha and beta giardin (Thompson and Weilinga, 2007).

However the glutamate dehydrogenase (GDH) gene has been useful in the genotypic analysis of *G. lamblia* isolates in mammals; the sequencing of this gene groups the isolates into all the sub genotypes AI, AII, BIII and BIV (Monis *et al.*, 1996). This factor is supported by the polymorphic nucleotides of GDH gene (Boontanom *et al.*, 2010).

The restriction fragment length polymorphism polymerase chain reaction (PCR-RFLP) is a reproducible, dependable and sensitive technique for genotyping *Giardia* (Read *et al.*, 2004). This method allows easy genotyping of *G. lamblia* isolates from humans, dogs and livestocks and also gives important information on the genotype in a population.
Studies on the diarrhoeal cases among hospitalized children, using questionnaire administration to identify associated risk factors (age, gender, place of residence, presence of domestic animals, source of drinking water and food), clinical features and molecular studies provided a good information on the prevalence of giardiasis (Mukherjee et al., 2009; Bello et al., 2010).

Previous studies in Ghana on intestinal parasites (including *G. lamblia*) focused on the types of parasites present and its prevalence as shown in studies by Addy et al., 2004; Reither et al., 2007 and Opintan et al., 2010. There is therefore the need for the establishment of the genotypes of *G. lamblia* present in Ghana, in order to determine how each of these genotypes impacts on the symptoms of infection. This information will enhance effective management of the infection. This study seeks to provide information on the prevalence of *G. lamblia* and its associated genotypes present through molecular epidemiology study.

1.2 Rationale of the study

The exposure of Ghanaian children to diarrhoea causing infectious agents is mostly through the use of contaminated water and unhygienic practices in food preparation and poor excreta disposal practices (Demographic and Health Survey, 2008). The prevalence of intestinal infections transmitted through the oral-fecal route has been reported to be high (21%) among food vendors in Accra. Of this *G. lamblia* accounted for 2.0% (Ayeh-Kumi et al, 2009). This tends to pose a risk to children, especially those who live in the tropical regions (including Ghana) where socio-economic standards are low, access to portable water is limited, personal and environmental hygiene is poor. Additionally, lack of knowledge about parasite transmission, overcrowding, environmental faecal contamination and inadequate sanitation standards put children at risk (Stanley and Reed,
2001; Stephenson et al, 2002 and Obeng et al, 2007). *Giardia lamblia* was the most prevalent intestinal parasite identified in stool samples of children in the Ashanti-Akim municipality (Nkrumah and Nguah, 2011), where socio-economic standards could be low.

Even though some studies have assessed the prevalence of diarrhoeal infections in Ghana, not much information has been gathered on giardiasis. Little is known about the hospitalisation of persons with giardiasis or its clinical features in Ghana. Also, no data exist on the genotypes of *G. lamblia* assemblages in Ghana. Information from this work could thus prove useful towards the effective treatment and control of the disease.

1.3 Aim

The aim of the study was to identify the genotype(s) or assemblage(s) of *G. lamblia* following their identification in the stool samples of children at age five or below.

1.3.1 Specific objectives

1. To estimate the prevalence of *G. lamblia* infection in children with symptomatic diarrhoea.
2. To genotype *G. lamblia* isolates among positive respondents.
3. To determine any association between age and sex of infected children and the identified genotypes of *G. lamblia*.
4. To determine the association between symptoms such as temperature/fever and vomiting and *G. lamblia* genotypes present amongst children with symptomatic diarrhoea.
CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The Disease

Giardiasis is a waterborne disease caused by a unicellular flagellated protozoan, *Giardia lamblia* in humans. It is a non-invasive parasite that undergoes binary fission on the epithelial cells of the small intestine of its host (Adam, 2001).

Antonie von Leeuwenhoek first discovered the parasite in 1961 in his stool. Initially it was not thought to be pathogenic until in the past two decades when data accumulated showed that the organism is a parasite and does exist in both epidemic and sporadic disease forms (Heresi and Cleary, 1997). The infection is transmitted through the oral-faecal route and people become infected when they ingest the cyst of the parasite. The cyst is mostly contained in contaminated food, water or by direct contact with infected persons (Thompson and Monis, 2004).

About 200 million people have symptomatic giardiasis in Asia, Africa and Latin America with 500,000 new cases being reported each year (WHO, 1996). The prevalence of asymptomatic giardiasis in people in the developed countries is between 2% and 5% while that of developing countries ranges between 20% and 30%. The rate of infection is higher in children than in adults (Oyerinde et al., 1977). Human infections with *G. lamblia* may result in either chronic or acute disease symptoms including diarrhoea and mal-absorption, however the differences in these symptoms is contributed by the strain of the parasite, the immune response of the host and the number of times one has been exposed to the parasite (Aggarwal and Nash, 1987; Farthing, 1996; Gottstein et al., 1990).
2.1.1 Epidemiology of Giardiasis

2.1.1.1 The Disease Distribution Worldwide

Giardiasis is distributed worldwide with infection rates being higher in developing countries than in developed countries. It is more prevalent in children than in adults (Dennis et al., 1993). The disease is globally distributed because of the low inoculum size, resilience of the cyst and a wide diverse of animal hosts (Heresi and Cleary, 1997). Giardiasis and other intestinal parasitic diseases have a high prevalence within people with improper personal hygiene, inappropriate sewage and garbage disposal system, unsafe drinking water and deprived environmental cleanliness (Adamu et al., 2006 and Noor-Azian et al., 2007).

In most developing countries, G. lamblia infections are common among children and may therefore threaten their growth and development. The prevalence among children in endemic areas mostly peaks at age 2 to 3 years and declines with increase in age (Nunez et al., 1999; Escobeda et al., 2007). Escobedo et al., (2008b) also revealed that Cuban children under 6 years of age were three times more at risk for G. lamblia infections than those aged between 6 to 10 years. High prevalence has also been recorded in children at day care centres and primary schools. The reasons for the high prevalence of G. lamblia infection in children could be due to immaturity of the body’s immune system, the infection being primary and their body could lack the ability to restrain discharge (faeces) voluntarily. Other factors include low personal hygiene, regular person-to-person contact and regular hand and mouth contact (Rojas et al., 2008).

In a hospital based study on children with giardiasis by Escobedo et al. (2010) the prevalence was higher in boys than in girls. This result also agrees with a study conducted by Keiser et al., (2002) in a community in Cote d’Ivoire where the prevalence in males
was twice that of females. This could possibly be due to the fact that most male children are more adventurous which exposes them to the risk of being infected by the parasite. However in a study by Nunez et al., (1999) there was no difference in terms of prevalence between males and females.

2.1.1.2 The Diseases Distribution in Ghana

Nkrumah and Nguah (2011) conducted a study on the childhood diarrhoea status in a Ghanaian district hospital and results showed that the general incidence of all parasites was 114 per 1000 with *G. lamblia* being the most common (89.5%) parasite. The study also showed that the prevalence of the infection increases with the age of the children. Klaus et al., 2007 also studied on the causes of acute diarrhoea in the northern region of Ghana and results showed that *G. lamblia* was the most common intestinal parasite even though its prevalence was low. The reason suggested was that stool sample collection was done in the dry season (November- January). The prevalence of the parasite was also higher in asymptomatic patients (5%) as compared to patients with symptomatic diarrhoea (13%). Also in a study on symptomatic patients in Ghana gave 3.7% prevalence for *G. lamblia* parasite infection (Addy et al., 2004).

2.2 Taxonomic Classification of *Giardia*

*Giardia* spp belongs to Kingdom Protozoa, Phylum Metamonada, Class Trepomonadacea, Order Diplomonadida, Family Hexamitidae and Genus *Giardia* (Cavalier, 1993). The distinguishing feature that is used to separate the genus *Giardia* from other members of the family Hexamitidae is the presence of a ventral adhesive disc. The ventral adhesive disc is supported by cytoskeleton of microtubules, microfilaments and fibrous structures. The disk is also composed of tubulin and other similar proteins called giardins (Crossley
and Holberman, 1983; Peattie et al., 1989; Alonso and Peattie, 1992). Other proteins present in the disc include actin, myosin and trypomysin (Freeley et al., 1982).

Three species of the *Giardia* genus that were initially identified include *G. agilis* in amphibians, *G. muris* in rodents and *G. duodenalis* were first described by Filice, 1952. Three other species identified include *G. psittaci* which is common in parakeets (Erlandsen and Bemrick, 1987), *G. ardae* which is common in herons (Erlandsen, 1990) and *G. microti* mostly found in muskrats and voles (Van Kuelen et al., 1998).

### 2.3 Pathogenensis and Pathology of Giardiasis

The pathology of giardiasis includes the excystation of cyst, attachment of trophozoites to the epithelial barrier and interference of ion secretion in the small intestine. Even though a greater percentage of infected individuals may show no symptoms (Rodriguez-Hernandez, 1996). The disease pathogenesis and severity is dependent on the parasite virulence factors and the immune and nutritional status of the host (Solaymani-Mohammadi and Singer, 2010).

There are direct morphological changes in the intestinal epithelial cells as a result of some products released by the parasite (Buret et al., 2002b). These interfere with the uptake of fat and other fat-soluble vitamins. There is also a decrease in the production of intestinal mucosal disaccharide enzymes (Heresi and Thompson, 1997). The increase in the permeability of the epithelial cells leads to an inflammatory response and both digestive and absorptive changes that correlate with brush border injury, disaccharidase deficiencies, parasite-induced apoptosis and sometimes allergic disorders (Chin et al., 2002; Scott et al., 2002).
2.4 Clinical features of the disease

The major symptoms of giardiasis include mild to moderate abdominal ache, abdominal distension due to increased intestinal gas, acute to chronic diarrhoea and weight loss (Heresi and Cleary, 1997). Other symptoms of giardiasis are chronic or acute diarrhoea, abdominal pains, abdominal cramps, nausea, flatulence, bloating, vomiting and fever. Dehydration, weight loss and anorexia have also been indicated (Heresi and Thompson, 1997).

Stools are normally soft but not watery, bulky and have a stinking smell. They also look greasy, bubbly and they float on the surface of water. Other symptoms that may occur include nausea, weakness, loss of appetite and impairment of growth, especially in children. Giardiasis may be suspected if the symptoms last for 7 days or more (Cheesebrough, M (2006).

2.5 Clinical diagnosis of *Giardia lamblia* infection in humans

The methods that have been used so far for diagnosing *G. lamblia* in stool samples is through microscopy, enzyme linked immunosorbent assay (ELISA) and the polymerase chain reaction (Cheesebrough, M 2006; Rockwell, 2003 and Sulaiman et al., 2003).

2.5.1 Morphological detection of *G. lamblia*

Microscopy is the traditional method for diagnosis where the cyst of the parasites can be seen under the microscope in a stool sample. Identification of the cyst of *G. lamblia* in fecal specimen can be done either through direct stool smear or by using the formol-ether concentration technique. Both techniques are fast but the direct stool smear is faster. The formol-ether concentration technique is more advantageous because it concentrates most of the parasites contained in stool samples. Because the cysts of the parasites are shed
irregularly, several specimens may need to be examined using the microscopy technique (Cheesebrough, M 2006).

2.5.2 Immunological detection of *Giardia lamblia* antigen

The use of Enzyme Linked Immuno Sorbent Assay detects *Gairdia* spp. antigens in the stool samples, the presence which indicates active infection. The use of the immunoassay has about 92.6% relative sensitivity and 98.1% relative specificity (Cheesebrough, M 2006). The results from a study by Janoff *et al.* (1989), suggested that ELISA may be more sensitive to than microscopy which is the reference standard for detection. They also concluded that the use of antigen detecting method provides an adequate and suitable adjunct to microscopy.

2.5.3 Molecular detection of *Giardia lamblia*

Further advances into molecular research studies in epidemiology has resulted in the use of a more useful (highly specific and sensitive compared to the others such as microscopy and ELISA) technique which is the polymerase chain reaction (PCR). This technique amplifies any loci of interest in any genome for further studies. It allows amplification of nucleic acid sequences both for the purposes of disease, pathogen detection and also for the sequencing of templates (Bemmingham and Leuttich, 2003).

This technique has been very useful in the epidemiology of giardiasis (Robertson *et al*., 2006) and has also provided more insight into the zoonotic importance of *G. lamblia* (Monis and Thompson, 2003). The PCR-restriction fragment length polymorphism (PCR-RFLP) has been used in the identification of the glutamate dehydrogenase gene which provides detailed information on both assemblages A and B of *G. lamblia* (Read *et al*., 2004). The restriction digest enzyme *NlaIV* was used for the detection of the assemblages
AI, AII, BIII and BIV and will be used in this study. The RsaI enzyme was used to digest amplicons for the detection of the assemblages BII and BIII (Boontanom et al., 2010). The deoxyribonuclease acid was extracted from fecal samples in most studies (Read et al., 2004; Bertrand et al., 2005; Boontanom et al., 2010). The DNA that was also used in this study was extracted from fecal samples from humans.

2.6 Prevention of Giardiasis

Giardiasis is a diarrheal disease and preventive measures recommended by WHO includes the following;

Good breastfeeding and weaning practices: It is advisable that babies should be breastfed exclusively during the first six months of their lives. Infants that are breastfed exclusively have a low tendency of getting diarrhoea or dying from it. When weaning a child, good weaning practices are encouraged to help protect the child from diarrhoea. These include the provision of nutritious food, preparing the infants meal under hygienic conditions and feeding the child with clean spoon. Others include washing of uncooked food with clean water before feeding the infant with it and covering of food that is being kept. It is also recommended that foods that have been kept for a long time must be re-heated before the child with it (WHO, 2003).

The use of safe water: Most diarrhoeal infections are transmitted through the consumption of contaminated water. The most hygienic accessible should be used. These water sources must also be protected from other sources of contamination. The sources of bathing, washing and defecation must be sited more than 10 metres from the houses. Places where water is stored must be done in clean and covered containers. Water kept in these containers must be changed frequently. Animals must be prevented from drinking from the sources of water. Children must be prevented from playing and defecating in water bodies.
People whose water are not safe for immediate consumption must be boiled to make it safe (WHO, 2003).

Handwashing: All infectious agents that cause diarrhoea can be transmitted by hands that are contaminated with fecal materials. The practice of handwashing must therefore be encouraged among both children and adults. Handwashing is required especially after visiting the toilet, before preparing meal and before eating. Effective handwashing requires the use of good soap and clean water (WHO, 2003).

2.7 Treatment of the Giardiasis

There are different classes of chemical agents with different chemical properties used to in the treatment of giardiasis. The major group involved is the nitroimidazoles; these include metronidazole, ornidazole, tinidazole and secnidazole. These drugs were initially used for the treatment of other protozoans parasites such as *Trichomonas vaginalis* and *Entamoeba histolytic* (Durel et al., 1960; Tracy and Webster, 1996). However a study by Darbon *et al.*, (1962) showed that metronidazole commonly known as flagyl is the most effective against *G. lamblia*. It is the most recommended drug for the treatment of giardiasis worldwide (Bost, 1977; Gardner and Hill, 2001). A study by Tracy and Webster (1996) showed that it is easily assimilated into tissues of the body. The daily-recommended dosage in children is 5mg/kg twice in a day (Gardner and Hill, 2001). The administration of metronidazole in a single dosage has about 36 to 60% effectiveness in a day; it increases to between 67 to 80% in 2 days dose and ranged between 80-100% when administered over 5 to 10 days in both adult and children. The drug usually comes in a tablet form but a liquid suspension can be made from it by mashing the tablet in glycerin and cherry syrup (Gazder and Banerjee, 1978; Jokipii and Jokipii, 1978; Tracy and Webster, 1996). The adverse effects for metronidazole include dizziness, vomiting and
headache. Other adverse effects include the infection of the pancreas, severe pains in the intestines and the central nervous system may be affected (Levi et al., 1977; Kusumi et al., 1980; Roe, 1985)

The other members of the nitroimidazole group of drugs (ornidazole, secnidazole and tinidazole are also recommended because their half lives are longer as compared to metronidazole and are therefore good for single dose therapy (Rosssignol, 1985). In a study conducted by Anderson et al., (1972) tinidazole also known as fasigyn was successfully used to treat patients with an efficacy range of 80-100% with a single dose of 2g. Liquid suspension of tinidazole is available, making it suitable for children at a single dose of 50mg/kg (Farthing, 1996; Pengsaa, 1999).

Quinacrine is also a drug of choice in the treatment of giardiasis even though it was initially an antimalarial drug. Its clinical efficacy has been estimated to be about 90% (Wolfe, 1975; Tracy, 1996). The recommended dosage in children is 6mg/kg per day taken in three doses in a day over five to seven day period (Lerman and Walker, 1982). Adverse effects in children include vomiting, headache and skin discoloration to yellow or pale orange. These have lead to the reduction in the efficacy of quinacrine in children below the age of 5 (Craft et al., 1981).

Furazolidone or Furoxone is another drug effective in the treatment of *G. lamblia* infections. The recommended dosage in children is 2mg/kg, twice a day for 10 days while the adverse effects may include nausea, diarrhoea and brown discoloration of urine (Gardner and Hill, 2001).

Other drugs of interest are Paromocycin, Albendazole and Bacitracin zinc. The recommended daily dosages for Paromocycin and Albendazole in children are 30mg/kg/day between 5 to 7 days and 15mg/kg/day between 5 to 7 days respectively.
Bacitracin zinc has not been recommended in children below 10 years. The adverse effect of Paromomycin may cause toxicity of the kidney. Albendazole may cause constipation (Gardner and Hill, 2001).

2.8 Biology of the parasite

*Giardia lamblia* has two separate developmental stages which are different in terms of structure and biochemistry; these are the cyst and the trophozoite stages (Adam, 2001). The motile pear-shaped dorsally convex trophozoite causes the clinical manifestation of giardiasis. The trophozoites before their maturation are contained in a cyst consists of a trophozoite that is surrounded by a protective cyst. The transmissible stage of the parasite is the one that eventually develops to cause giardiasis (Heresi and Thomas, 1997; Adam, 2001).

2.8.1 The trophozoite stage

This is the vegetative stage of the parasite and it resembles the human face in stained preparations. It ranges between 12µm- 15µm in length, 5µm- 7µm in width and 1µm-2µm in thickness (Heresi and Thomas, 1997 Adam, 2001; Solari *et al.*, 2003). It has two nuclei that lie in symmetry. The nucei are surrounded by nuclear envelopes. Four pairs of flagella emanates from each trophozoite. Each pair emerges from the anterior-lateral, ventral, posterior and caudal positions from the cell body (Heresi and Thomas, 1997; Lujan *et al.*, 1997; Elmendorf *et al.*, 2003). The flagella of the trophozoite are made of microtubules from the basal bodies between the nuclei. The main use of the flagella is to aid in movement of the trophozoite. It is also important in the attachment of the parasite to the epithelial cells of the small intestine (Ghosh *et al.*, 2001).
The antero-ventral side of the trophozoite is covered by the ventral disk. This is connected to the plasma membrane by fibres that are made of alpha and beta tubulin and contractile proteins. There are also cytoskeletal proteins collectively known as giardins in the trophozoites (Palm et al., 2005). The ventral disk is the most significant organelle among organisms of the genus *Giardia* (Elmendorf et al., 2003). It functions as a sucking disk that aid in the transfer of nutrient from the epithelial micro villi of the upper small intestine by diffusion (Solari et al., 2003; Benchimol, 2004).

Other organelles that are present in *G. lamblia* include the endoplasmic recticulum and other vesicles such as the excystation-specific secretory vesicle. This sends components for cyst wall formation from the interior of the cell to its exterior. The cytoplasm also contains other vesicles that function as endosomes and lysosomes (Lujan et al., 1997; Adam, 2001; Gottig et al., 2006). The golgi apparatus, mitochondria and peroxisomes are absent. Also found in the cell are lysosome-like peripheral vacuoles located underneath the plasma membrane and a very complex cytoskeleton (Adam, 2001). A study conducted by Piva and Benchimol, (2004) on the median body of *G. lamblia* showed that the median body is both transversal and perpendicular to the central axis in the parasite. The study suggested the median body to function as a reserve for microtubules and is also important in the biogenesis of the ventral disc.

**2.8.2 The Cyst Stage**

The cyst stage of *G. lamblia* is composed of a trophozoite that is encompassed by a rigid protective cyst. This enables the parasite to survive outside its host. The cyst also helps the trophozoite to overcome extreme environmental conditions. The cyst stage is the means by which the parasite infects to its host (Lujan et al 1997; Adam, 2001). The cyst can thrive in
cold temperatures for between 60 to 90 days and can be heat killed at a temperature of about 50°C (Heresi and Thompson, 1997).

The cyst is ovoid in shape. Its length and width range between 8µm and 12µm and 5µm to 10µm respectively. The thickness of the cyst cell wall is between 0.3µm and 0.5µm. Biochemically, the cell wall is made of carbohydrates (mainly N- acetyl galactosamine) and other cyst wall proteins.

Figure 1: Trophozoite of *Giardia lamblia*.

Source: University of Western Ontario (www.instruct.uwo.ca/biology/332a/WK1)
2.9 Life cycle of the parasite

*Giardia lamblia* infections occur in humans directly by the oral-fecal route or through the consumption of food and water contaminated by the cyst of the parasite (WHO, 2003). The incubation period of the parasite is usually between 5 and 25 days, but in acute infections it is usually between 5 to 6 days (Hunter, 1998). When the parasite reaches the small intestine, each cyst goes through a stage of excystation. This process results in the release of two trophozoites. The trophozoites undergo further longitudinal binary fission resulting in the release of several trophozoites in the proximal part of the small intestine. Here they remain attached to the mucosal lining by its ventral sucking disc. As the trophozoites travel down the large intestine, they go through another period of encystations. This results in the formation of cysts around them. The cysts are common in both diarrhoeal and non-diarrhoeal stools.
Figure 2: The life cycle of *Giardia lamblia*.

Source: Centre for disease control and prevention (www.dpd.cdc.gov/dpdx)

2.10 Molecular characterization of *Giardia lamblia*

*Giardia lamblia* is the only species of *Giardia* that has been isolated from humans. It also has a zoonotic potential because it has also been isolated from other mammals (Weilinga and Thompson, 2007). The use of molecular tools has lead to the detection of different genotypes (assemblages) of *G. lamblia*. Five assemblages of this parasite have been detected. Assemblages A and B have been solely isolated in humans and other vertebrates.
Assemblages C and D have been isolated in dogs and assemblage E was isolated in hoofs. Assemblage F was isolated in cats and G in rats and dogs (Weilinga and Thompson, 2007).

Further molecular studies have lead to the detection of sub-genotypes of *G. lamblia*. The genotype A has been sub-divided into AI and AII while genotype B has sub-genotypes BIII and BIV (Adams 2001; Monis *et al.*, 2006). Various polymorphic genes have been targeted for the characterization of *G. lamblia* in most studies. These include the small sub-unit ribosomal DNA (SSU rDNA), variable surface protein (vsp), glutamate dehydrogenase (gdh), triosephosphate isomerase (tpi), elongation factor 1 alpha (ef1-α) and beta giardin (β giardin) (Weilinga and Thompson, 2007). Studies by Berilli *et al.* (2012), found the human assemblages A and B of *G. lamblia* in humans and also in dogs, goats, ducks and chicken. Assemblages C and D were only found in dogs. This provides good evidence on the potential transmission of zoonotic pathogens to humans from free-range animals in close relations.

### 2.9.1 The Glutamate Dehydrogenase enzyme and the Glutamate Dehydrogenase Gene (GDH gene)

Glutamate dehydrogenase is an enzyme that is involved in the reversible reaction for converting glutamate to α-ketogluterate and vice versa in the urea cycle (Spariaki and Plaitakis, 2012). This enzyme is encoded by the glutamate dehydrogenase gene. GDH gene has been of great use in genotyping *G. lamblia* isolates in eukaryotes because of its high polymorphic nature. Through PCR-RFLP, all four sub-genotypes (AI, AII, BIII and BIV) isolated from humans can be distinguished with the use of GDH gene (Monis *et al.*, 1996). Initially the sensitivity of the nested PCR for the amplification of the GDH gene had a lower sensitivity (53%) especially in the presence of lower number of cysts. An
improved primer sets were developed and used by Boontanom et al., 2010 which had a sensitivity of 83% as compared to the one developed by Read et al., 2004. The primers for nest 1 are GDH1 (ATC TTC GAG AGG ATG CTT GAG)-forward, GDH1a (ATC TTC GAG AAG GAT GCT TGA G)-forward and GDH5s (GGA TAC TAC TTS TCC TTG AAC TC)-reverse. The primers for nest 2 were GDHeF (TAC ACG TYA AYC GYG GYT TCC GT)-forward and GDHiR (GTT RTC CTT GCA CAT CTC C)-reverse. In the above sequences, Y could be Cytosine or Thymine and R could be either Arginine or Guanine (Boontanom et al., 2010). These primers when used was able to amplify 19 (95%) out of 20 isolates of G. lamblia. The expected diagnostic band size was 461 base pairs (Boontanom et al., 2010).

2.11 Risk factors affecting Giardiasis transmission in children

*Giardia lamblia* has emerged as the most ubiquitous intestinal parasite in humans, some domestic and wild mammals worldwide (Thompson and Monis, 2004). Thus it occurs in both developed and developing countries. In both situations children are known to be more at risk of giardiasis than in adults (Mahdy et al., 2008). The risk of acquiring the disease is mostly associated with the socio-demographic, hygiene, nutritional and immune status of the host as well as the strain of the parasite (Thompson, 2000; Sackey et al, 2003; Mahdy et al, 2008).

In most developed countries the risk of infection is highly associated with the consumption of contaminated tap water, fresh water (swimming pools through recreational activities of swimmers) and the movement of individuals from a non-endemic region to an endemic region (Isaac-Renton and Philion, 1992; Hoque et al., 2002). Studies conducted by Hoque et al., (2003) among children in New Zealand, revealed that changing of nappies in children was associated with a high risk of giardiasis.
In developing countries the predominant risk factor associated with *G. lamblia* infection are socio-demography. These include improper sanitation, bad personal hygiene, eating of raw fruits and vegetables and drinking of contaminated tap water (Stuart *et al.*, 2003; Mahdy *et al.*, 2008). In a study by Mukherjee *et al.*, (2009) at Kolkata in India, there was a relationship between giardiasis and the socio-economic background of the study population. Their results showed that most of the diarrhoeal patients had lower socio-economic status, the study population lived in the slums, so the disease condition could be highly associated to water and food borne contaminations. Children whose ages were less than or equal to five years were most at risk for parasitic infections.

Most studies have revealed that *Giardia lamblia* is less frequent in adults than children (Mahmud *et al.*, 1995) and that in children; it is more likely to be symptomatic (Islam *et al.*, 1983). Nunez *et al.*, (1999) in their study revealed that the incidence of giardiasis in three day care centres in Havana, Cuba peaked between the ages of two and three. The incidence of giardiasis increased with increasing age. The proposed reasons could be due to acquired immunity or improved standard of hygiene.

Reddaiah *et al.*, (1999) conducted a study on the behaviour of gender discrimination in India to access the effect of sociobiological factors on child mortality. Results showed that almost 77% of 286 deaths occurred among infants. Of this 77%, majority of them were females whose ages were below one year. Mahmud *et al.*, (1995) also did a cohort study in Rural Egypt and their results showed that *G. lamblia* is more likely to occur in female infants as compared to male infants. Thus the males had lower number of symptomatic infection. The reason provided was that some socio-cultural and economic regulations could be operating in Egypt that keeps the male infants from encountering microbes in the
environment. This includes the fact that males are given special attention and care than females.

2.12 Immune responses generated from *G. lamblia* infection

Both cell-mediated and humoral immune responses have been shown to be effective in most protozoan infections. Humoral immunity is more active in blood stages of most protozoan infections while cellular immunity is more active when cells of the host have been infected (Kuby, 2000). Most microbial pathogens of which *G. lamblia* is not an exception do undergo antigenic variation which is characterized by the constant switching process of one variant of surface protein to another. This allows the parasite to escape the immune defense of its host and may result in chronic infections (Nash, 2002; Deitsch *et al.*, 2009).

The symptoms of giardiasis may persist in individuals for weeks, months or even years. This depends greatly on the immune status of an individual. In healthy individuals infection is mostly cleared within one to three weeks (Rendtorff, 1957). On the other hand, a study by Chester *et al.*, (1985) proposed that *G. lamblia* infections may remain in individuals who are immunoglobulin A deficient or in immunocompromised individuals. Istre *et al.*, (1984) studied the evidence of acquired immunity in water borne giardiasis at a Ski resort in Colorado, the study revealed that people who have already acquired giardiasis are less likely to be ill when re-infected. This is because they have become immune to the disease. Isaac-Renton *et al.*, (1994) also suggested a similar reason for observations in their study on second outbreak of water borne giardiasis and serological investigations in Canadian patients. Immunoglobulin A has been known to provide immune protection against giardiasis. The patients with defective B cells usually have chronic giardiasis (Stark *et al* 2009).
CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Chemical, Reagents and Equipments
The sources and manufacturers of reagents, buffers, solutions and equipments used in the study are listed in Appendix I

3.2 Study Site
The study was conducted at the Princess Marie Louise Childrens’ Hospital in Accra, Ghana. This health facility is the main pediatric specialist hospital in Ghana where kwashiorkor a child malnutrition was first described. The hospital has 75 bed capacity and is located within the Korle Wokon district a heavily populated community in Accra. The hospital serves a population of diverse demographic characteristics; the main diseases reported at the facility are malaria, diarrhoea, upper respiratory infections, malnutrition and HIV/AIDS. The main indigenes of this district are Gas whose major occupation are fishing for the men and fish mongering for the women. Nevertheless due to rural-urban migration there is a great mix-up in the population with majority being traders. The major source of drinking water is tap water and sachet water.

3.3 Study design and Study Population
A hospital-based cross sectional study was conducted at the Princess Marie Louise Hospital. All children aged 0-60 months presenting symptoms of diarrhoea at the hospital that fulfilled the criteria for enrollment as participants of the study within the period of six months (August 2012-January 2013) were recruited. Informed consent was sought from the parents or guardian of participants after they had been briefed about the purpose of the study. Both demographic and bio-data of participants were also obtained through the
administration of questionnaires Stool samples were collected from only participants whose parents gave consent. Stool samples were collected into labelled sterile stool containers using collecting spoons in each container.

The quantity of the fresh stool samples obtained was within a range of 1000 to 1500 mg. These were transported in a cooler with ice packs to the Parasitology Department of the Noguchi Memorial Institute for Medical Research for analysis. In the laboratory, about 300mg of each stool sample was collected from the container and stored at -20°C in a freezer for molecular analysis. Deoxyribonucleic acid (DNA) was extracted from 200mg of each of the stored samples. Each of the extracted DNA was amplified through a nested polymerase chain reaction (PCR) and DNA fragment sizes determined by agarose gel electrophoresis. All samples that were positive for *G. lamblia* were further digested with endonucleases and assessed to obtain the genotypes present. The genotypes recorded where confirmed by DNA sequencing.

3.4 Ethical Considerations

Ethical approval for this study was sought from the Institutional Review Board at School of Allied Health of the University of Ghana.

3.5 Inclusion criteria

All hospitalized children between the ages of 0-60 months (August - January) presenting with diarrhoea within a period of six months were recruited into the study. Written and oral informed consent was sought from the parents or guardians of the children before they were allowed to participate in this study.
3.6 Exclusion criteria

All children between the ages of 0-60 months without symptomatic diarrhoea and children whose parent did not consent were not allowed to participate in the study.

3.7 Sample size estimation

A study by Addy et al., (2004) on the prevalence of parasites in hospitalized infants with diarrhoea reported the prevalence of *G. lamblia* to be 3.7% in Ghana. Assuming a minimum prevalence of 5% for *G. lamblia* infection, a minimum sample size of 72 will be obtained at a 95% confidence interval of width ±5%. A twenty percent allowance was made to compensate for sample loss or any such eventualities. In all, a sample size of 145 participants was arrived at and used in this study.

The sample size was estimated using the formula;

\[ n_o = \frac{Z^2 P(1-P)}{d^2} \]

(Cochran et al., 1977)

In the formula stated above, \( n_o \) is the required sample size. \( Z \) is the confidence level at 95%, which is a standard value of 1.96. \( P \) is the estimated prevalence in the project area and \( d \) is the margin of error. The margin of error used was 5% at 95% confident interval.

3.8 Questionnaire Administration

Demographic and biometric information were obtained from the parents or guardians who consented for their wards to be participants. Clinical data and all other information obtained through questionnaire administration were recorded by the health attendants at the hospital.
3.9 Samples from respondents

The parents of all children who fulfilled the inclusion criteria were given a labelled sterile stool container with a collecting spoon in it for sample collection. This enabled collection of stool samples for laboratory analyses.

3.10 Laboratory Studies

The stool samples were transported to the Parasitology Department of the Noguchi Memorial Institute for Medical Research for DNA extraction and molecular identification.

3.10.1 Sample processing

A single stool sample was collected from each child. The fresh stool samples were transported to the laboratory. In the laboratory about 300mg of the stool sample were kept in labelled 1.5ml Eppendorf tubes and stored in the freezer at -20°C for later molecular studies.

3.10.2 Molecular Studies

Deoxyribonucleic acid was extracted from stool samples and amplified using primers specific for the characterization of *G. lamblia* in a modified nested PCR methods of Read et al., (2004) and Boontanom et al., (2010). A restriction enzyme digest was carried out on identified *G. lamblia* using modified methods of Boontanom et al., (2010)

3.10.2.1 Genomic DNA extraction from stool sample

The UltraClean® Fecal DNA Isolation Kit (MOBIO Laboratories, Inc. /Catalog No. 12811-100) was used to extract the parasite DNA from 250µg frozen stool sample. The
extraction was done according to the manufacturer’s protocol. The active components of the reagents were not disclosed by the manufacturers.

The stored stool samples at -20°C were allowed to thaw at room temperature before use. Two hundred and fifty microliters of the stool sample was pipetted into the 2µl dry bead tube (the beads in the bead tube is for lysing the cells in the fecal matter) and 550µl of bead solution (a buffer solution) was added to each. Each tube was vortexed gently for even mixture of its contents and 60µl of pre-warmed solution S1 (contains Sodium dodecyl sulphate, detergent that hastens cell lysis by breaking down the lipids and fatty acids of the cell membrane) was added. The tubes were inverted gently to mix content very well. Two hundred microliters of the inhibitor removal solution (it precipitates PCR inhibitors) was added to the mixture and the tube was vortexed at 13.2 rotations per minute for 10 minutes. The supernatant was then transferred from the bead tube into a clean 2ml collection tube. Two hundred and fifty microlitres of solution S2 (a protein precipitation reagent that removes contaminating proteins) was added to the supernatant, vortexed for 5 seconds and incubated at 4°C. After 5 minutes the tubes were retrieved and centrifuged at 10,000 revolutions for 1 minute. Four hundred and fifty microlitres of the supernatant from each tube was pipetted into a new 2µl Eppendorf tube, 900µl of solution S3 (a DNA binding salt solution that facilitates the binding of DNA to the silica membrane) was added to it and vortexed for 5 seconds. Into a spin filter was pipetted 500µl of the mixture and centrifuged a 10,000 revolutions for a minute. This step was repeated until all the mixture was filtered through the spin filter. Three hundred microlitres of solution S4 (an ethanol based wash solution used to further clean the DNA that is bound to the silica membrane and to remove salt residues from solution S3) was pipetted into the spin filter and centrifuged at 10,000 revolutions for 30 seconds. The solution that flowed through the spin filter was discarded and the spin filter was centrifuged at 10,000
for one minute. The spin filter was then placed in a new 2µl eppendorf tube and 50µl of solution S5 (Tris [hydroxymethyl aminomethane], a sterile elution buffer) was pipetted into the spin filter and centrifuged at 13.2 (maximum speed) for 30 seconds. The extracted DNA was stored at -80°C.

3.10.2.2 PCR amplification

Two sets of primers designed from the DNA sequences of *G. lamblia* were used for the molecular identification using diagnostic DNA fragment sizes (Boontanom *et al.*, 2010). The sequence details of the primers and the expected band sizes of the PCR products are given in Appendix 2. A nested PCR was performed on the DNA eluted from the stool samples using a protocol by Boontanom *et al.*, (2010) with modifications.

The nest 1 PCR reaction mix of 25µl contained 10X PCR buffer supplied by the manufacturer (Sigma, USA), 0.5µM of each of the four deoxyribonucleotide triphosphates (dNTPs), 1.25µM of each oligonucleotide primers, 0.75µM magnesium chloride and 0.2 units of *Taq* Polymerase enzyme (Sigma USA). Three microlitres of the genomic DNA was used as a template for the amplification reaction and double distilled sterile water was added to make up the volume. The reaction mix was centrifuged for 10 seconds and the DNA amplification carried out using a 2720 thermal cycler (Applied Biosystems, USA). The cycling parameters for the reactions were as follows: 94°C for 7 minutes (initial denaturation), followed by 35 cycles of 94°C for 1 minute (denaturation), 55°C for 1 minute (annealing), 72°C for 1 minute (extension) and then a final extension at 72°C for 5 minute.

The nest 2 PCR reaction mix of 25µl contained 10X PCR buffer supplied by the manufacturer (Sigma, USA), 0.5µM of each of the four deoxyribonucleotide
triprophosphates (dNTPs), 1.25µM of each oligonucleotide primers, 0.75µM magnesium chloride and 0.2 units of Taq Polymerase enzyme (Sigma USA). One microlitre of the PCR product from nest 1 was used as a template for the amplification reaction and sterile double distilled water was added to make up the volume. The reaction mix was centrifuged briefly. The amplification was carried out using a 2720 thermal cycler (Applied Biosystems, USA) with the cycling parameters for the reactions as follows: 94°C for 2 minutes (initial denaturatin), followed by 55 cycles of 94°C for 30 seconds (denaturation), 56°C for 20 seconds (annealing), 72°C for 45 seconds (extension) and a final extension at 72°C for 5 minutes.

The visualization of the nest 2 product is indicated in section 3.10.2.4 of this chapter.

For each set of reaction, a positive control containing PCR product of G. lamblia of the same primer set, as the template and a negative control that contained no DNA was included.

3.10.2.3 Molecular forms of G. lamblia genotype identification

The presence of G. lamblia in stool samples were identified using the nested PCR with the genotypes present being further determined using the restriction enzyme NlaIV (BIOLABS INC, NEW ENGLAND) in a restriction enzyme digest. The recognition site for Nla IV is GGN^NCC. The restriction fragment length polymorphism (RFLP) technique aided in the determination of the genotypes present (Boontanom et al., 2010).

The NlaIV enzyme digestions were carried out using the recommended protocol given by the manufacturers (BIOLABS INC, NEW ENGLAND). A final reaction volume of 20µl contained 10µl of amplified product, 0.2µl of 1x Bovine saline albumin (BSA), 2µl of 1x NlaIV, 2µl of 1x reaction buffer and 5.8µl of sterile double distilled water. After
preparation of the reaction mix, incubation at 37°C for 3 hours followed to allow for digestion of the amplified DNA fragments. Electrophoresis of the digested DNA fragments was carried out by running the product together with a loading dye orange G through a 2% agarose gel stained with ethidium bromide. Visualization of the digested product was photographed in UV light illumination as described in section 3.10.2.4 of this chapter.

3.10.2.4 Observation and analysis of amplified PCR-RFLP products

The amplified products of *G. lamblia* were electrophoresed separately on 2% agarose gel stained with 0.5µ/ml ethidium bromide. Five microlitres of each sample was added to 1µl of orange G (5x) gel loading dye and loaded into each well of the agarose gel for electrophoresis. The gel was prepared and electrophoresed in 1X TAE buffer using a mini gel system (BIO RAD, USA) at 100 volts for 50 minutes. The gel was photographed using a UV trans-illuminator (UPC, USA) fitted with a short wavelength polaroid camera containing film type 667 (Polaroid, USA). The sizes of the PCR products were estimated by comparison with the mobility of a 100 base pair molecular marker (Sigma p-1473, see Appendix I) with the expected diagnostic sizes of the PCR amplified fragments of the different genotypes as stated in Appendix II.

3.10.2.5 Sequencing, Sequence analysis and Phylogenetic clustering

Four of the *G. lamblia* PCR (40µl) positive products were sent for purification and sequencing (Macrogen Inc., Netherlands). The products were sequenced in only the forward direction with the forward primer (GDHeF) for all loci. The sequences were then compared to other sequences from the gene bank using the NCBI Blast Local Alignment Search Tool (BLAST) to find regions of local similarity between sequences (Altschul *et al.*, 1990 and [http://blast.ncbi.nlm.nih.gov/Blast.cgi](http://blast.ncbi.nlm.nih.gov/Blast.cgi)). Sequence similarity to database
sequences were based on expect value (E), maximum identity and score, query coverage and total score. Multi-sequence alignments and phylogenetic analyses were performed with MEGA V5 (Tamura et al., 2011). The trees in which the associated taxa clustered evolutionary in history were inferred using the Neighbor-Joining method (Saitou and Nei, 1987) with the optimal tree having the sum of branch length as 0.37134009. The percentages of replicate together in the bootstrap test (1000 replicates) were shown (Figure 6) next to the branches (Felsenstein, 1985). The tree was drawn to scale (Figure 6), with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of base differences per site. The analysis involved 7 nucleotide sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

3.11 Data Analysis

The data obtained were non-parametric, nominal and ordinal. All the data were analyzed using version 17 of SPSS programme for windows (SPSS Inc., Chicago, IL, USA). The best statistical analysis was the Chi square ($\chi^2$) to test the associations between two variables. The data was analyzed to determine the association between giardiasis and age, sex and symptoms of the disease (vomiting, fever and diarrhoea). The significance was determined as $p<0.05$. 


CHAPTER FOUR

RESULTS

4.1 Characteristics of study participants

A total number of 145 hospitalized participants within the age ≤ 60 months were recruited for this study. All participants presented with diarrhoea and their age range was ≤ 48 months with majority of the participants being ≤ 36 months. The mean age of respondents was calculated to be 14.54± 10.89 months (standard deviation) and the age distribution is shown in figure 3. There were 88 males and 57 females involved. Results from the clinical data on other symptoms (fever, temperature and vomiting) associated with diarrhoea are shown in table 1. The duration of diarrhoea was between 1 to 5 days whiles that of vomiting was between 1 to 6 days. Ambient temperature range during the study was between 30°C to 40.2°C.
Figure 3: Age distribution of participants recruited for the study.
Table 1: Study sample age and sex composition

<table>
<thead>
<tr>
<th>Factor</th>
<th>Number involved</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 12 months</td>
<td>86</td>
<td>59.3</td>
</tr>
<tr>
<td>13-24 months</td>
<td>38</td>
<td>26.2</td>
</tr>
<tr>
<td>25-36 months</td>
<td>10</td>
<td>6.9</td>
</tr>
<tr>
<td>37-48 months</td>
<td>11</td>
<td>7.6</td>
</tr>
<tr>
<td>49-59 months</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>88</td>
<td>60.7</td>
</tr>
<tr>
<td>Females</td>
<td>57</td>
<td>39.3</td>
</tr>
</tbody>
</table>

Source: Personal data collected at the Princess Marie Louise Hospital, Accra.

4.2 Prevalence of *Giardia lamblia* using PCR

Overall percentage prevalence of *G. lamblia* in stool samples of hospitalized children attending the Princess Marie Louise Hospital was 6.2%. Of the 145 samples analyzed, 9 were positive and 136 were negative for *G. lamblia*. Majority of the positive samples (66.7%) were ≤ 12 months. The percentages of positives were greater in males than in females. Tables 2 and 3 respectively show the total percentage prevalence and age and sex distribution of prevalence. No statistically significant association was observed between *G. lamblia* infection and the age grouping (P(χ²) >0.05 [Table 3]).
Table 2: Percentage *G. lamblia* infections determined after sample examinations

<table>
<thead>
<tr>
<th></th>
<th>Number of samples</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positives</td>
<td>9</td>
<td>6.2</td>
</tr>
<tr>
<td>Negatives</td>
<td>136</td>
<td>93.8</td>
</tr>
<tr>
<td>Total</td>
<td>145</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 3: Chi square ($\chi^2$) analysis of the effect of age group and sex on the prevalence of *Giardia lamblia*

<table>
<thead>
<tr>
<th>Factor</th>
<th>Number involved</th>
<th>Number of positives (%)</th>
<th>$\chi^2$ value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 12 months</td>
<td>86</td>
<td>6(66.7)</td>
<td>4.918</td>
<td>0.178</td>
</tr>
<tr>
<td>13-24 months</td>
<td>38</td>
<td>1(11.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25-36 months</td>
<td>10</td>
<td>2(22.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>37-48 months</td>
<td>11</td>
<td>0(0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>49-59 months</td>
<td>0</td>
<td>0(0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>88</td>
<td>8(88.9)</td>
<td>3.198</td>
<td>0.74</td>
</tr>
<tr>
<td>Females</td>
<td>57</td>
<td>1(11.1)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.3 The symptom of Diarrhoea and its duration recorded among study participants

As mentioned previously, all patients (145) recruited for this study had diarrhoea. The range for diarrhoea duration was between 1 to 5 days while the mean duration for diarrhoea was calculated to be 2.14±1.17 (standard deviation) days respectively. Amongst those positive for the *G. lamblia* infection (9/145), the highest number (44.4%) was recorded on the first day of admission for diarrhoea with the numbers decreasing as the study progressed in days. Here also no significant association was found between *G. lamblia* and the duration of diarrhoea was not statistically significant. (P value of 0.585 [Table 5]).

Table 4: Duration of Diarrhoea among participants (Days)

<table>
<thead>
<tr>
<th>Day</th>
<th>Number involved</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>57</td>
<td>39.3</td>
</tr>
<tr>
<td>2</td>
<td>37</td>
<td>25.5</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>20.7</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
<td>10.3</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>4.1</td>
</tr>
<tr>
<td>Total</td>
<td>145</td>
<td>100.0</td>
</tr>
</tbody>
</table>
Table 5: Chi square analysis of the association between duration of diarrhoea and *G. lamblia* percentage infections (%)

<table>
<thead>
<tr>
<th>Day</th>
<th>Number of subjects</th>
<th>Percentage <em>G. lamblia</em> infection among study subjects (%)</th>
<th>$\chi^2$ value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>145</td>
<td>4 (44%)</td>
<td>2.840</td>
<td>0.585</td>
</tr>
<tr>
<td>2</td>
<td>37</td>
<td>3(33%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>1 (11%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>15</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>1 (11%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>-</td>
<td>9 (100)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.4 The symptom of Vomiting and its duration recorded among participants of this study

The total number of participants that vomited were 113 (77.9%). Majority of the participants vomited on Day 2 (31.0%) of admission and the number decreased in the course of the study. There was no statistically significant associated between *G. lamblia* infection (P=0.454) and the period/day of vomiting as well. (P = 0.165. [Table 6 and 7]).
Table 6: Percentage number of participants that vomited and the period/day of vomiting and the percentage positives for *G. lamblia* infection

<table>
<thead>
<tr>
<th>Period/day</th>
<th>Number involved</th>
<th>Percentage (%)</th>
<th>$\chi^2$ value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No vomiting</td>
<td>32</td>
<td>22.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vomitting</td>
<td>113</td>
<td>77.9</td>
<td>0.56</td>
<td>0.454</td>
</tr>
<tr>
<td>Day 1</td>
<td>29</td>
<td>25.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 2</td>
<td>35</td>
<td>31.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 3</td>
<td>31</td>
<td>27.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 4</td>
<td>13</td>
<td>11.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 5</td>
<td>4</td>
<td>3.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 6</td>
<td>1</td>
<td>0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>145 (100)</td>
<td></td>
<td>113</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 7: Duration of Vomitting among *G. lamblia* positives (%) participants

<table>
<thead>
<tr>
<th>Day</th>
<th><em>G. lamblia</em> positives (%)</th>
<th>$\chi^2$ value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4 (44%)</td>
<td>9.147</td>
<td>0.165</td>
</tr>
<tr>
<td>2</td>
<td>3 (33%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1 (11%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1 (11%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>9 (100)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.5 Body temperature and cases of fever among study participants

The minimum and maximum body temperatures recorded for the study participants were 35.0°C and 40.2°C respectively with the mean temperature being 37.5±1.05°C. Among those positive for *G. lamblia* infection, 55.6% had fever and 44.4% had no fever. There was no significant association between *G. lamblia* and fever. (P value = 0.812 [Table 8]).

![Figure 4: Percentage prevalence of fever amongst study participants.](http://ugspace.ug.edu.gh)
Table 8: Association between *Giardia lamblia* infections and having fever among participants

<table>
<thead>
<tr>
<th></th>
<th>Number involved (%)</th>
<th>$\chi^2$ value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fever</td>
<td>5 (55.6)</td>
<td>0.52</td>
<td>0.812</td>
</tr>
<tr>
<td>No fever</td>
<td>5 (44.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>9 (100)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* Fever: Temperature reading $\geq 37^\circ$C  
*b* No fever: Temperature reading $< 37^\circ$C

4.6 Molecular identification of *Giardia lamblia* isolates from stool samples of participants

One hundred and forty-five samples were examined for *G. lamblia* isolates through PCR. Nine samples were successfully amplified as *G. lamblia* at band size of 461 base pairs (Figure 5).
Figure 5: An ethidium bromide-stained agarose gel (2%) electrophoregram of amplified PCR products for the identification of G. lamblia. (Lane 1 is the molecular weight marker [100bp ladder], lane 2-10 are participants’ samples, lane 11: positive control and lane 12: negative control).
4.7 Restriction fragment length polymorphism (RFLP) of Genotypes and Sequence identification

Table 9 shows the RFLP analysis profiles of *G. lamblia* isolates from nine samples used in this study. Digestion of the PCR amplification products with *NlaIV* enzyme yielded two DNA fragments of band sizes 120 and 290 base pairs indicating that they are all genotype B isolates. The phylogenetic clustering of study isolates is depicted in figure 6 while figure 7 shows an ethidium bromide stained gel of *NlaIV* restriction digest of *G. intestinalis*.

Table 9: The RFLP profiles of *G. intestinalis* isolates

<table>
<thead>
<tr>
<th>Restriction enzyme</th>
<th>RFLP profiles</th>
<th>Genotype</th>
<th>Identified species</th>
<th>Number of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>NlaIV</em></td>
<td>120 base pairs, 290 base pairs</td>
<td>B</td>
<td><em>G. intestinalis</em></td>
<td>9</td>
</tr>
</tbody>
</table>

The RFLP could not discriminate between subgenotypes for genotype B (which are BIII and BIV) therefore four samples were selected from the nine that were positive for *G. intestinalis* by RFLP for sequencing. BLAST analysis confirmed all four to be *G. intestinalis* of the genotype B. Phylogenetic analysis suggested one sample (N15408 GDHe348) as subgenotype BIII.
The reference sequences, *G. intestinalis* GDH, *Giardia intestinalis* H22 GDH (BIV), *G. intestinalis* MQG95 GDH (BIII), *G. psittaci*, and *G. ardeae* were retrieved from GenBank with accession numbers AB195224, EF507665, JQ700429, AB714978 and AF069060 respectively. Isolates assessed in this study were GPML1037 GDHe440 and N15408 GDHe438, which clustered with reference *G. intestinalis* orthologs. However, N15408 GDHe438 clustered more clonally with *G. intestinalis* MQG95 GDH (BIII), suggesting they are related species. A total of 222 positions were observed in the final dataset with the nucleotide sequence of sample N15408 shown in appendix VI.
Figure 7: An ethidium bromide-stained gel (2%) electrophoregram of NIAIV restriction enzyme digest for the identification of genotypes of *G. lamblia*. (Lane 1: molecular weight marker (100-bp ladder); lanes 2 to lane 6 are participants’ samples and lane 7: negative control.)
CHAPTER FIVE

DISCUSSION

5.1 Discussion of results

The prevalence of *G. lamblia* infection and its genotype was investigated among participants between the ages of 0-5 years who were hospitalized for diarrhoea. Since diarrhoea is the major symptom of giardiasis (Herens and Cleary, 1997), the prevalence of acute childhood diarrhoea among age this age group was investigated.

Acute diarrhoea is a major cause of mortality and morbidity among children in developing countries especially in children ages 0-5 years (Kosek *et al.*, 2003). In the current study 92.4% of the total respondents were among ages 0-36 months (table 1). This result is similar to findings from a study by Addy *et al.*, (2004) which also showed a high prevalence of diarrhoea among these age groups with a prevalence of 88.9%. Olusanya *et al.*, (1994) also recorded 98.4% of the children within this age group in their study in Nigeria to have diarrhoea. This suggests that most children within the ages of 0-36 months are frequently exposed to agents that cause diarrhoea probably as a result of their nurturing and the nature of their immediate environment. Also their immune system is not matured to protect them since their exposure to the risk factors may be early. All respondents that had *Giardia lamblia* infection were among ages 0-36 months in the current study (table 3).

Analysis of the association between age and sex and the genotype of *G. lamblia* recorded, resulted in a P value >0.05 (table 3). This shows that there was no significant association between age, sex and the genotype of *G. lamblia* present.

According to a study conducted by Klaus *et al.*, (2007) in northern Ghana, the prevalence of *G. lamblia* in symptomatic participants with diarrhoea was 3.7%. Also in a study conducted by Addy *et al.*, 2004 the prevalence of *G. lamblia* was 3.7% among infants (0-5
years) with diarrhoea. However, the prevalence of *G. lamblia* infections in respondents was 6.2% for the current study (table 2). This result was almost twice as much as that obtained in studies by Klaus *et al.*, (2007) and Addy *et al.*, (2004). One main reason could be that the methods for detection of the parasite used in the present study differ from that of the afore-mentioned studies. Thus molecular technique of PCR was used to detect the parasite in this study compared to enzyme linked immunosorbent assay (ELISA) and microscopy used in the other studies.

The genotype characterization of *G. lamblia* is important in understanding the disease dynamics in epidemiological studies and outbreaks of infections. It helps in the detection of the sources of infection and identification of the appropriate treatment required (Robertson *et al.*, 2006) which was one of the aims of this study. The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) is a reliable and sensitive method that allows the detection of even the lowest level of infections that other techniques such as microscopy is unable to do (McClade *et al.*, 2003). It has been used to differentiate between *G. lamblia* isolates from assemblages A and B successfully in most studies (Read *et al.*, 2004; Homan and Mank, 2001). This useful technique was therefore employed in this study. The glutamate dehydrogenase (GDH) gene has been useful as a genetic marker in the characterization of human *G. lamblia* isolates in most studies because of its high polymorphism. The sequencing of this gene locus has aided in the grouping of human isolates into both assemblages A and B and sub- assemblages AI, AII, BIII and BIV (Read *et al.*, 2004). This gene was examined in this study to differentiate between giardia isolates. The genotype detected from all the *G. lamblia* positive isolates from this study after the PCR-RFLP was genotype B (Table 9). However, PCR-RFLP did not differentiate between the sub-genotypes BIII and BIV since the restriction enzyme used (*Nla IV*) classifies both sub-genotypes at the same band sizes of 120 and 290 base
pairs (Read et al., 2004). Although human isolates of *G. lamblia* have been characterized into either genotype B or A (Read et al., 2004; Monis et al., 2003) studies by Monis et al., (1999) showed that genotype B tends to be human specific. The results obtained from this study agree with results obtained from other studies (Mahdy et al., 2009; Monis et al., 2003). There is no published data on the genotypes of *G. lamblia* in Ghana; however it has been reported in different countries. A study by Mahdy et al. (2009) in Malaysia reported genotype B to be more predominant in all human stool samples while genotype A was found in only one sample among the clinical cases that reported with diarrhoea.

Sequence analysis of four of the nine PCR-RFLP isolates was confirmed to be indeed genotype B with a high sequence similarity of about 98% (Appendix VI). Separately, phylogenetic analysis showed clustering of all isolates with reference *Giardia intestinalis* orthologs. Two isolates used in this study to construct the phylogenetic tree (Figure 6) were GPML 1037 GDHe440 and N15408 GDHe438. However, N15408 GDHe438 clustered more clonally with *Giardia intestinalis* MQG95 GDH (BIII), suggesting they are related species. This observation is consistent with studies by Monis and Thompson, (2003). Additionally, data from the tree topology in this study agrees with that of Monis and Thompson, (2003) which suggests that both assemblages A and B evolved from *G. ardae*.

No significant association was observed between most symptoms associated with gastroenteritis (diarrhoea, vomiting and fever) and genotype B (table 5, 6 and 8). P values were > 0.05 in all cases. This suggests that the children were not so much exposed to the *G. lamblia* parasite but other gastroenteritis causing agents. Hence the presence of gastroenteritis could be attributed to other diarrhoea causing agents. Clinical findings associated with giardiasis indicate an incidence of about 70% to 100% diarrhoea, 20% to 30% vomiting and 17% to 28% fever (Heresi and Cleary, 1997). However, participants in
this study with giardiasis displayed symptoms of 100% diarrhoea, 100% vomiting and 55.5% fever. According to Homan and Mank, (2001) a strong correlation exist between the symptoms (diarrhoea, fever, vomiting and abdominal cramps) recorded and the genotypes (A and B). Mahdy et al., (2009) also found a strong correlation between clinical symptoms of gastroenteritis and genotype B with a P = 0.019 in a study in Malaysia. Even though there was no correlation between the clinical symptoms (tables 5, 6, 7 and 8) and the genotype B in this study it could be suggested that most clinical cases of giardiasis could be caused by the genotype B isolates of G. lamblia. Mahdy et al., (2009) also gave a similar conclusion on their study among clinical cases of diarrhoea.
6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

The main finding of this study was that genotype(s) of *G. lamblia* encountered in Ghanaian children presenting with diarrhoea was Genotype B (III or IV). DNA sequence analysis confirmed one sample as genotype BIII. This could help in the development of drugs for the treatment of giardiasis among clinical cases. The prevalence of symptomatic giardiasis was 6.2% amongst infants with childhood diarrhoea in Ghana. There was no significant association between symptoms associated with gastroenteritis (fever, vomiting and diarrhoea) and the genotype recorded. Diarrhoea and giardiasis were more prevalent among children less than 36 months of age.

6.2 Recommendations

Questionnaires on investigations on the major risk factors (presence of domestic animals, sources of food, sources of drinking water, breastfeeding habits and the level of education of parents or guardians) would have provided more information on the risk factors that exposes one to giardiasis. The sample size should be increased to minimise errors. Asymptomatic individuals should be included in future studies to see if other genotypes will be present amongst them.
REFERENCES


http://www.dpd.cdc.gov/dpdx Centers for Disease Control (CDC) assessed on 20th January, 2012 at 13:30 hrs GMT


Trophozoite of *G. lamblia* assessed on 15th October, 2011.
APPENDICES

APPENDIX I

PREPARATION OF STANDARD SOLUTIONS

With standard sterile double distilled water (sdd H₂O), the following standard solutions were prepared and autoclaved at 121 lb/sq for 15 minutes in an Eyela autoclave (Rikikaki, Tokyo).

Primers for PCR

Primers used in PCR reaction were reconstituted and diluted according to the manufacturers recommendations (Viracor-IBT Laboratories, USA).

Preparation of Electrophoresis buffer

Electrophoresis buffer, Tris-acetate (TAE), concentrated stock solution: 242g of Tris base was dissolved in 57.1 ml glacial acetic acid and 100 ml EDTA. pH was adjusted to 7.7 (with glacial acetic acid) and the volume made to 1000ml with double distilled water. The 50x solution was dispersed into aliquots and stored at room temperature. The working solution was 1x.

Preparation of Ethidium Bromide

Ethidium bromide, 10mg/ml: 0.1g of ethidium bromide was added to 10ml of distilled water and stirred on a magnetic stirrer for several hours to ensure the dye dissolved very well. The container in which the solution was stored was wrapped with aluminium foil and
stored at room temperature. Gloves were worn during the preparation of this solution because it is carcinogenic.

**5X Orange G (Gel loading buffer)**

20% (w/v) Ficoll was added to 25 mM EDTA and 2.5 % (w/v) orange G were used. 0.25g of Orange G was dissolved in a few millilitres of sterile distilled water and 500µl of 5M EDTA was added. A final volume of 10ml was obtained by the addition of distilled water. It was stored at room temperature.

**DNA molecular weight size markers**

100bp DNA molecular weight size marker obtained from the manufactures (Sigma) was diluted according to the recommendations and used; 10µl of DNA ladder, 30µl of loading dye (orange G), 60µl of sddH₂O. For the 100bp ladder, the first band size is 100bp, subsequent ones are read 200, 300, 400, …………………1000bp.

**2% Agarose gel**

First 0.8g of agarose powder was put into flask and TAE was added to make a volume of 40ml. It was heated in microwave oven 230V, 50Hz, 2660W, 12.0A) for 1 minute to dissolve solute. The mixture was cooled under water and 2µl of Ethidium Bromide was added. The gel was cast to set in a chamber with comb to make the wells.
APPENDIX II CONSTITUENTS OF A – PCR REACTION MIX USED IN THE NEST 1 OF MOLECULAR STUDIES OF GIARDIA LAMBLIA DNA

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile double distilled water</td>
<td>14.3</td>
<td></td>
</tr>
<tr>
<td>10X PCR buffer</td>
<td>2.5</td>
<td>X1</td>
</tr>
<tr>
<td>50mM MgCl$_2$</td>
<td>0.75</td>
<td>1.5mM</td>
</tr>
<tr>
<td>10mM of each dNTP (dATP, dCTP, dGTP, dTTP)</td>
<td>0.5</td>
<td>200µM</td>
</tr>
<tr>
<td>10µM of each primer</td>
<td>1.25</td>
<td>500nM</td>
</tr>
<tr>
<td>5U Taq polymerase</td>
<td>0.2</td>
<td>1U</td>
</tr>
<tr>
<td>DNA template</td>
<td>3</td>
<td>1ng-1µg</td>
</tr>
</tbody>
</table>
## APPENDIX III CONSTITUENTS OF A – PCR REACTION MIX USED IN THE NEST 2 OF MOLECULAR STUDIES OF GIARDIA LAMBLIA DNA

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile double distilled water</td>
<td>17.55</td>
<td></td>
</tr>
<tr>
<td>10X PCR buffer</td>
<td>2.5</td>
<td>X1</td>
</tr>
<tr>
<td>50mM MgCl₂</td>
<td>0.75</td>
<td>1.5mM</td>
</tr>
<tr>
<td>10mM of each dNTP (dATP, dCTP, dGTP, dTTP)</td>
<td>0.5</td>
<td>200µM</td>
</tr>
<tr>
<td>10µM of each primer</td>
<td>1.25</td>
<td>500nM</td>
</tr>
<tr>
<td>U Taq polymerase</td>
<td>0.2</td>
<td>1U</td>
</tr>
<tr>
<td>DNA template</td>
<td>1.0</td>
<td>1ng-1µg</td>
</tr>
</tbody>
</table>
APPENDIX IV
DNA EXTRACTION PROTOCOL

The stored stool samples at -20°C were allowed to thaw at room temperature before use. Two hundred and fifty microliters of the stool sample was pipetted into the 2µl dry bead tube (the beads in the bead tube is for lysing the cells in the fecal matter) and 550µl of bead solution (a buffer solution) was added to each. Each tube was vortexed gently for even mixture of its contents and 60µl of pre-warmed solution S1 (contains Sodium dodecyl sulphate, detergent that hastens cell lysis by breaking down the lipids and fatty acids of the cell membrane) was added. The tubes were inverted gently to mix content very well. Two hundred microliters of the inhibitor removal solution (it precipitates PCR inhibitors) was added to the mixture and the tube was vortexed at 13.2 rotations per minute for 10 minutes. The supernatant was then transferred from the bead tube into a clean 2ml collection tube. Two fifty microlitres of solution S2 (a protein precipitation reagent that removes contaminating proteins) was added to the supernatant, vortexed for 5 seconds and incubated at 4°C. After 5 minutes the tubes were retrieved and centrifuged at 10,000 revolutions for 1 minute. Four-fifty microlitres of the supernatant from each tube was pipetted into a new 2µl collection tube, 900µl of solution S3 (a DNA binding salt solution that facilitates the binding of to the silica membrane) was added to it and vortexed for 5 seconds. Into a spin filter was pipetted 500µl of the mixture and centrifuged a 10,000 revolutions for a minute. This step was repeated until all the mixture was filtered through the spin filter. Three hundred microlitres of solution S4 (an ethanol based wash solution used to further clean the DNA that is bound to the silica membrane and to remove salt residues from solution S3) was pipetted into the spin filter and centrifuged at 10,000 revolutions for 30 seconds. The solution that flowed through the spin filter was discarded and the spin filter was centrifuged at 10,000 for one minute. The spin filter was then
placed in a new eppendorf tube and 50µl of solution S5 (Tris [hydroxymethyl aminomethane], a sterile elution buffer) was pipetted into the spin filter and centrifuged at 13.2 (maximum speed) for 30 seconds. The extracted DNA was stored at -80°C.
APPENDIX V

CONSENT FORM AND QUESTIONNAIRE

CONSENT FORM

Title: Epidemiology, Molecular and Immunological Characterization of Giardiasis in Ghana

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 and Dora Obrempomah Oddei on 0243145451.

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 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.

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

Prevalence of *Giardia lamblia* in diarrhoeal stools

Dear Respondent,

This questionnaire is designed for a research work from the Department of Animal Biology and Conservation Science, University of Ghana, legon. 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………………

   Place of habitation: ……………………………

2. Sex       A. Male [  ]     B. Female [  ]

   Sample No…………

**Clinical Data**

Please describe your child’s illness

3. Diarrhoea  (Yes) (No)

4. Vomiting   (Yes) (No)

5. Fever      (Yes) (No)

(Childs temperature on admission ……………..°C )
Duration of symptoms before admission

6. Date onset of diarrhoea ..............................................

7. Duration of diarrhoea (days) ........................................

8. Duration of vomiting (days)............................................
APPENDIX VI

NUCLEOTIDES FROM SEQUENCE ANALYSIS

>N15408_GDHe438

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>GPML1443_GDHe201

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74
Sample: N15408 GDHe438
Giardia intestinalis isolate D39 glutamate dehydrogenase (gdh) gene, partial cds
Sequence ID: gi|343915472|gb|JF918512.1|Length: 434. Number of Matches: 1

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Sbjct 92  CAGATCTCTGAAGAAACTCCCTTACACGCTTCCGATTTGGAAGACCCAGGGCTTCCGAC 151

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