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Giardiasis in children attending daycare centers in Guatemala and the therapeutic potential of ganglioside

by

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Abstract

Intestinal enteropathy is a prevalent yet neglected aspect of child malnutrition involving chronic exposure to intestinal pathogens such as *Giardia* intestinalis. Gangliosides have therapeutic potential for the treatment of giardiasis and intestinal enteropathy. The objectives of the thesis were to 1. assess the *Giardia* carriage patterns in daycares in Guatemala, 2. compare anthropometric data between *Giardia* carriers and non-carriers, 3. compare sensitivity and specificity of 3 *Giardia* diagnostic methods (ProSpecT-*Giardia*-EZ-Microplate assay, RidaQuick *Giardia* dipsticks and microscopic analysis using the sucrose concentration gradient method). *Giardia* prevalence rates were high (close to 44%), most cases of infection were chronic (5-week study period), and malnutrition (lower weight-for-age Z-scores) may be associated to higher intensity of infection. ELISA was the most sensitive *Giardia* diagnostic test. Compared to ELISA, the microscopic and dipstick analysis had sensitivities of 53.6% and 60.7%, and specificities of 100% and 97.9%, respectively, within a set of 75 stool samples.

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ANOVA	Analysis of Variance
CD4+	CD4 positive T-lymphocytes
CD8+	CD8 positive T-lymphocytes
CDC	Centers for Disease Control and Prevention
CER	Ceramide
CeSSIAM	Center for Studies of Sensory Impairment, Aging and Metabolism
CND	Canadian Dollar
DFA	Direct Immunofluorescence
DG	Diglyceride
DNA	Deoxyribonucleic Acid
EIA	Enzyme Immunoassay
ELISA	Enzyme-Linked Immunosorbent Assay
ETEC	Enterotoxigenic *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
G2	Growth Phase 2
GlcCER	Glucosil-Ceramide
GG	Ganglioside(s)
GSA-65	*Giardia* Specific Antigen 65
GTQ	Guatemalan Quetzal
HAZ	Height-for-Age Z-score
IBD	Inflammatory Bowel Disease

Ig	Immunoglobulin
IL	Interleukin
INF	Interferon
LacCER	Lactosyl-Ceramide
LBSA	Lipid-Bound Sialic Acid
L:M	Lactulose:Mannitol
LPS	Lipopolysaccharide
LT	Leukotriene
NCHS	National Center for Health Statistics
NEC	Necrotizing Enterocolitis
NO	Nitric Oxide
NPV	Negative Predictive Value
Nu+/nu+	Nude mouse
O&P	Ovum and Parasite
OD	Optical Density
OR	Odds Ratio
ORT	Oral Rehydration Therapy
PAF	Platelet Activating Factor
PCR	Polymerase Chain Reaction
PG	Prostaglandin
PPV	Positive Predictive Value
RR	Relative Risk
SMLN	Superior Mesenteric Lymph Node

SOSEP	*Secretaria de Obras Sociales de la Esposa del Presidente*
TCR	T-Cell Receptor
TGF	Transforming growth factor
Th1	T-helper Type 1 Cells
Th2	T-helper Type 2 Cells
TNF	Tumor necrosis factor
WAZ	Weight-for-Age Z-score
WGA	Wheat Germ Agglutinin
WHO	World Health Organization
WHZ	Weight-for-Height Z-Score

CHAPTER 1: INTRODUCTION

1.1 The Impact of Intestinal Enteropathy on the Nutrition State of Children

1.1.1 Background

Intestinal enteropathy has been described in many ways. Tropical jejunitis is a term that was adopted by Rosenberg & Scrimsahw (1972) to describe the gastrointestinal enteropathy or subclinical malabsorption reported by several different authors. Tropical jejunitis is a disease state in which the small intestine undergoes deleterious morphological and functional changes in response to chronic intestinal infections (Fagundes-Neto *et al*., 1984). A similar condition of gastrointestinal enteropathy in infants and children has been described by Lunn (2000), with more emphasis on the role of inflammation. Rosenberg (2003) discusses the emergence of the naming and classification of Tropical jejunitis, which is thought to be a less symptomatic and less advanced form of Tropical sprue. In contrast, other authors consider both conditions as distinct diseases (Baker & Mathan, 1970; Fagundes-Neto *et al*., 1984). Tropical Sprue is thought to occur mostly in adults and is characterized by chronic diarrhea, malabsorption, intestinal lesions and low concentrations of certain micronutrients (vitamin B12, folate and iron) (Baker & Mathan, 1972). Throughout this paper 'Intestinal enteropathy' will be used to describe the disease state in which the small intestine is damaged from chronic inflammation due to chronic exposure to intestinal pathogens associated with unsanitary environments. Damage to the small intestine is a major contributor to malnutrition in children living in impoverished, unsanitary conditions. Intestinal enteropathy may occur in conjunction with diarrhea and inadequate nutritional intake.

1.1.2 Diagnosis

Intestinal enteropathy can be diagnosed from a biopsy of the small intestine to confirm damaged tissue (blunted villi, intraepithelial leukocyte infiltration or

crypt cell hyperplasia). Alternatively, the lactulose:mannitol (L:M) test is a non-invasive biomarker that measures intestinal permeability and absorption. A high L:M ratio (generally a value >0.05) indicates impaired intestinal function. Measures of intestinal inflammation may also be used to indicate intestinal enteropathy (ex. fecal lactoferrin, calpain or calprotectin) (Guerrant *et al*., 2008). A point to note is that the results of an intestinal biopsy demonstrating damaged tissue and/or an abnormally high L:M ratio do not only occur in intestinal enteropathy; other disease states cause similar (if not indistinguishable) results. Therefore, diagnosis must be done in the context of an unsanitary environment.

1.1.3 Prevalence

Intestinal enteropathy is not routinely screened and due to its lack of overt symptoms, may not be detected. Research in the Gambia, West Africa has demonstrated a consistently high prevalence of intestinal enteropathy in infants and young toddlers. Lunn (2000) has estimated that 95% of infants aged between 2 and 15 months have experienced intestinal enteropathy. The majority of all cases consist of prolonged, chronic intestinal enteropathy likely to persist into adulthood (Lunn, 2000). A review of studies mainly conducted between 1960-1985 indicated that intestinal damage consistent with the description of intestinal enteropathy was commonly found in adults and children in variety of geographically dispersed developing countries (Haghighi & Wolf, 1997).
The true prevalence of intestinal enteropathy among children in the developing world is unknown. Several published studies have measured the L:M ratio in infants and young children (Lunn *et al*., 1991; Sullivan *et al*., 1992; Goto *et al*., 1999; Filteau *et al*., 2001; Northrop-Clewes *et al*., 2001; Goto *et al*., 2002; Campbell *et al*., 2003; Campbell *et al*., 2003; Campbell *et al*., 2004; Chen *et al*., 2003; Williams *et al*., 2007; Vieira *et al*., 2008; Goto *et al*., 2009). The studies have consistently found L:M ratios above 0.05, indicating abnormal intestinal function. The majority of the studies have been conducted in Gambia. In other

regions, abnormal gut function was found in 30% of Guatemalan and 48% of Brazilian study participants. In all other studies the reported group mean for L:M ratio was much higher than 0.05 (range of 0.07-0.73, Table 1-1).

1.1.4 Etiology

Intestinal enteropathy in children living in impoverished countries is thought to be caused by chronic enteric infections associated with unsanitary living conditions (Fagundes-Neto *et al*., 1984; Haghighi & Wolf, 1997; Humphrey, 2009). Enteric infections do not always result in diarrhea (Lunn, 2000). The consequences of asymptomatic infections, particularly chronic cases, are beginning to receive greater recognition for their role in malnutrition (Solomons *et al*., 1993; Lunn, 2000; Guerrant *et al*., 2008). The list of potential pathogens is long and includes bacteria, viruses and parasites (Petri *et al*., 2008). Common prevalent pathogens that are likely contributors to intestinal enteropathy include *Giardia intestinalis* (Lanata *et al*., 1992; Thurnham *et al*., 2000; Lanata & Mendoza, 2002; Petri *et al*., 2008), enterotoxigenic *Escherichia coli* (ETEC) (Lanata *et al*., 1992; Lanta & Mendoza, 2002), *Helicobacter pylori* (Lunn, 2000), *Cryptosporidium* spp., *Entamoeba histolytica* (Petri *et al*., 2008) and *Campylobacter* spp. (Lanata et al., 1992; Lanata & Mendoza, 2002; Petri *et al*., 2008). Often, the causative agents have been described generically as fecal pathogens ingested as a result of improper sanitation (Fagundes-Neto *et al*., 1984; Haghighi & Wolf, 1997; Humphrey, 2009). Malnutrition is part of the etiology of intestinal enteropathy as it facilitates chronic infection by weakening host immune defenses and predisposes the small intestine to pathogenic changes.

1.1.5 Pathogenesis

Intestinal enteropathy results in impaired growth, deterioration in nutrition status and possibly an increased susceptibility to translocation of macromolecules,

endotoxins and invasive enteric pathogens. Diarrhea may also occur.
In intestinal enteropathy, the small intestine is damaged. There are flattened villi, crypt hyperplasia, increased intra-epithelial lymphocytes, a compromised intestinal barrier, decreased absorptive surface area, decreased digestive enzymes and a pro-inflammatory milieu (Fagundes-Neto *et al*., 1984; Haghighi & Wolf, 1997; Veitch *et al*., 2001).

Malnutrition can negatively impact intestinal health and contribute to intestinal enteropathy. Stanfield et al. (1965) described deleterious changes such as villi atrophy, loss of epithelial absorptive area and increased number of cells in the lamina propria in the upper small intestine of children with kwashiorkor (protein-energy malnutrition with edema). Studies investigating severely malnourished children from Gambia have described the most extreme cases as having 'flat celiac-like mucosa' (Sullivan *et al*., 1991; Lunn, 2000). Campbell *et al*. (2003) found that severely malnourished children had a higher ratio of pro-inflammatory cytokines (INF-γ and TNF-α) to regulatory cytokines (IL-10 and TGF-β) in the small intestine compared to Gambian control children. This imbalance in cytokines creates a pro-inflammatory intestinal environment. Micronutrient deficiencies (iron, zinc and vitamin A) may contribute to a compromised intestinal barrier (Berant *et al*., 1992; Chen *et al*., 2003).

A major consequence of intestinal enteropathy is impaired growth. Impaired growth and a compromised nutrition state can occur from impaired nutrient and energy digestion/absorption due to damage to the small intestine (Briend, 1990; Weaver, 1999; Guerrant *et al*., 2008). Diarrhea can occur as a consequence of malabsorption leading to losses in water, electrolytes and nutrients. Diarrhea can contribute to impaired growth particularly if the diarrhea is recurrent and persistent (Guerrant *et al*., 2008). Malnutrition increases the duration and severity of diarrhea episodes in children. Severely malnourished children may be refractory to normal drug treatment therapies for enteric pathogens (Sullivan *et*

al., 1991). Other factors contributing to impaired growth and nutrition include increased energy demands associated with chronic immunostimulation (Klasing *et al*., 1987; Briend, 1990; Solomons *et al*., 1993; Weaver, 1999) and anorexia associated with infection (Solomons *et al*., 1993; Guerrant *et al*., 2008). The factors involved in the pathogenesis of intestinal enteropathy are intertwined and contribute to a vicious cycle leading to malnutrition, impaired growth and sometimes diarrhea.

1.1.6 Current treatment strategies

Treatment for intestinal enteropathy includes administering drug treatment for a particular pathogen(s), alleviating malnutrition, minimizing inflammation or administering particular nutrients designed to repair and maintain the integrity of the small intestine (Guerrant *et al*. 2008). On a broader level, strategies designed to improve environmental contamination factors (handwashing education, provision of toilets and water, elimination of poverty) could also help improve intestinal enteropathy (Fagundes-Neto *et al*., 1984). Humphrey (2009) points out that studies that have assessed the impact of environmental interventions have only monitored the impact on diarrhea and not intestinal enteropathy. Therefore the effectiveness of such strategies on improving intestinal enteropathy is not known. In many situations, chronic re-infection with various intestinal pathogens will likely occur due to the child's environment. Drug treatment targeted at pathogens is only effective short term. Recurrent drug treatment is not optimal as many medications have negative side-effects, may be toxic with chronic usage and contribute to drug resistance (Rosenberg *et al*., 1974). Chronic administration of low-dose antibiotic treatment to children does not result in long-term improvements in growth, and therefore its impact on intestinal enteropathy would also be questionable (Guzman *et al*., 1958). Treating malnutrition through increased energy intake without alleviating intestinal inflammation or healing the small intestine is also inefficient and not completely effective (Sullivan *et al*.,

1992). The most effective solution is to repair and maintain the small intestine in conjunction with an improved diet that will enable the child to achieve a desirable nutrition state resulting in a strengthened immune system so that they may be prepared to ward off future infections (Guerrant *et al*., 2008). Reducing pathogen exposure through improved environmental conditions and appropriate home hygiene is also essential for long-term recovery (Rosenberg *et al*., 1974). Guerrant *et al*. (2008), Duggan *et al*. (2002) and Ziegler *et al*. (2003) summarized nutrients with potential benefits for gastrointestinal conditions including intestinal enteropathy.

1.1.6.a Glutamine and Arginine

Glutamine and arginine are conditionally essential amino acids. A conditionally essential amino acid signifies that under optimal conditions the body can synthesize that particular amino acid from precursors. However, under certain conditions of excessively high energy demands or restrictive intakes, such as infection or chronic malnutrition, the body cannot synthesize sufficient quantity and therefore becomes relatively deficient in that amino acid (Duggan *et al*., 2002; Ziegler *et al*., 2003; Guerrant *et al*., 2008).

Glutamine supplementation may help improve intestinal enteropathy by reducing damage to intestinal cells, promoting intestinal cell proliferation and repair, and maintaining the intestinal barrier. Glutamine is an important energy source for cells with high turnover rates such as intestinal mucosal and immune cells (Bulus *et al*., 1989; Scheppach *et al*., 1994; Wiren *et al*., 1998; Labow & Souba, 2000; Ziegler & Daignault, 2000). Glutamine may help to reduce intestinal damage (oxidation) because it is a precursor to the antioxidant compound glutathione (Jones, 2002). Glutamine deficiency may result in impaired mucosal turnover and a compromised intestinal barrier (Labow & Souba, 2000; Ziegler & Daignault, 2000). Glutamine supplementation may help to maintain the intestinal barrier by

reducing toxin-induced damage to tight junctions and adherence junctions (Seth *et al*., 2004; Seth *et al*., 2004). Glutamine stimulates crypt cell proliferation (Rhoads *et al*., 1997) and is necessary for epidermal growth factor-induced intestinal cell proliferation (Ko *et al*., 1993). Intestinal cell proliferation is an important step for regular renewal of the epithelial layer as well as intestinal repair (epithelial restitution) that occurs when the small intestinal is damaged (Sturm & Dignass, 2002).

Glutamine may be additionally effective at treating cases of intestinal enteropathy that also result in diarrhea. Glutamine promotes electrolyte and water absorption helping to reduce diarrhea morbidity (Bushen *et al*., 2004) and is successful in Oral Rehydration Therapy (ORT) during acute diarrhea (Ribeiro *et al*., 1994). Results from a clinical trial demonstrate that glutamine supplementation (0.3 g/kg body wt per day) reduces the duration of diarrhea (Yalcin *et al*., 2004).
To date, there have not been many clinical trials investigating the effect of glutamine supplementation at reducing intestinal enteropathy. Glutamine supplementation has been shown to reduce infection in humans in clinical settings (Ziegler *et al*., 1992; Gianotti *et al*., 1995; Zhang *et al*., 1995; Neu *et al*., 1997). Glutamine is thought to decrease bacterial translocation in the gastrointestinal tract resulting in a reduced occurrence of sepsis (Duggan *et al*., 2002). Animal data are consistent with findings that glutamine supplementation may improve L:M ratios (Li *et al*., 1994). Serum glutamine concentrations were reported to correlate with L:M ratios and growth in Brazilian children with intestinal inflammatory disorders, however, further details were not given (Guerrant *et al*., 2008). Glutamine supplementation for a period of 10 days improved the L:M ratio in a pediatric population in Brazil (Lima *et al*., 2005). The children in this study also received a dose of vitamin A. In contrast, glutamine supplementation (0.25 g/kg body weight per day for a period of 5 months) did not improve the L:M ratio or growth in young Gambian infants compared to control (Williams *et al*.

2007). The authors suggested that the lack of effect was possibly due to an insufficient dose.

Arginine supplementation may be beneficial to intestinal enteropathy by promoting more rapid regeneration of mucosal cells (Wu *et al*., 2000). One of the mechanisms of arginine supplementation is from its role as a precursor for nitric oxide (NO). NO is necessary for local blood flow to the small intestine. Increased or adequate local blood flow could be beneficial for delivery of oxygen and nutrients to recovering or regenerating cells. However, NO can also cause damage systemically through excessive inflammation and increased intestinal vascular permeability (Kubes & McCafferty, 2000; Duggan *et al*., 2002). Arginine may play a protective role in patients at high risk of intestinal disease associated to trauma, critical illness or cancer (Duggan *et al*., 2002). However, most studies have used arginine supplementation in combination with other treatments making it difficult to elucidate a specific effect from arginine. Animal studies have shown that arginine supplementation resulted in improved mucosal recovery and intestinal barrier function after small bowel resection or radiation treatment to the small intestine (Welters *et al*., 1999; Ersin *et al*., 2000). Necrotizing enterocolitis (NEC) is an intestinal condition mainly affecting premature infants consisting of excessive inflammation and a compromised intestinal barrier (Duggan *et al*., 2002). Oral arginine supplementation reduced histological damage and lipid peroxidation of intestinal cells in a mouse model of NEC (Akisu *et al*., 2002). Human premature infants supplemented with arginine had a lower prevalence of NEC compared to control (Amin *et al*., 2002). These findings suggest that arginine supplementation may be beneficial at improving various intestinal diseases, particularly diseases associated with inflammation or requiring regeneration of intestinal cells. However, more clinical trials investigating the effects of arginine supplementation at improving intestinal enteropathy are needed.

1.1.6.b Zinc

Zinc is a potential treatment agent for intestinal enteropathy because it has antioxidant properties, helps to maintain the intestinal barrier, is important for mucosal cell renewal, is necessary for optimal immune function and is effective at reducing diarrhea (Shankar & Prasad, 1998; Powell, 2000; Lukacik *et al*., 2008). Duggan *et al*. (2002) highlight the importance of zinc for cells with a high turnover rate such as enterocytes and immune cells.

Zinc is part of the structure of superoxide dismutase, an enzyme complex with potent antioxidant capabilities (Virgili *et al*., 1999; Wapnir, 2000). Zinc can quench free radicals formed by transition metals (mainly iron and copper) and prevent oxidation of certain sulfhydryl groups present on proteins (Powell, 2000). Zinc promotes antioxidant systems indirectly through formation of metallothioneins (Powell, 2000). Metallothioneins are a group of proteins that bind metals and their presence in tissue (including intestinal tissue) is associated to reductions in damage caused by oxidation (Powell, 2000). Zinc deficiency results in mucosal atrophy (Vanderhoof *et al*., 1986), reduced mucosal cell turnover (Wapnir, 2000), impaired antigen presentation and T-cell function in the gut-associated lymphoid tissue (Shi *et al*., 1998), impaired phagocytosis and cytokine production (Shankar & Prasad, 1998), and facilitates survival of intestinal nematodes in rodent models of infection (Koski & Scott, 2001). Mucosal atrophy impairs water and electrolyte absorption (Ghishan, 1984; Wapnir, 2000) and contributes to malabsorptive diarrhea (Wapnir, 2000).
A meta-analysis of clinical trials has concluded that zinc supplementation decreases the duration of diarrhea (acute and persistent diarrhea) and the number of diarrhea stools passed per day (Lukacik *et al*., 2008). Zinc supplementation (alone or in combination with vitamin A) may also improve the L:M ratio in children (Alam *et al*., 1994; Chen *et al*., 2003). Zinc supplementation in animals is associated with improved tight junction structure and function (Rodriguez *et al*.,

1996). However, further evidence of the effect of zinc supplementation at improving intestinal enteropathy is needed including more insight into the specific mechanism of action (Duggan *et al*., 2002).

1.1.6.c Vitamin A

Vitamin A is a lipid-soluble vitamin, which can exist under different isomeric forms and is considered to include retinol, retinaldehyde, retinoic acid, retinyl ester and the carotenoids (Basu & Dickerson, 1996). Vitamin A is essential for normal morphology of epithelial cells including the mucous lining of the gastrointestinal tract (Basu & Dickerson, 1996). Lack of vitamin A causes keratinization, which the epithelial layer becomes dry, rough and fibrous due to the presence of keratin in the cells, an insoluble protein (Basu & Dickerson, 1996).

Data from animal studies indicates that vitamin A regulates crypt cell proliferation (Wang *et al*., 1997) and differentiation (Ruifrok *et al*., 1996). Vitamin A impacts the number of goblet cells present in the crypts and villi of the small intestine (Rojanapo *et al*., 1980; Warden *et al*., 1996). Prolonged vitamin A deficiency results in decreased intestinal villus height, decreased disaccharidase activity (Warden *et al*., 1997) and can make the intestine more prone to inflammatory damage (Ruifrok *et al*., 1996; Warden *et al*., 1997) or damage from pathogens (Ahmed *et al*., 1990; Warden *et al*., 1997). Vitamin A may be important for intestinal repair because it is necessary for intestinal adaptation following small bowel resection (Swartz-Basile *et al*., 2000). Vitamin A may also attenuate intestinal damage through its role as an antioxidant (Ziegler *et al*., 2003). Vitamin A may promote maintenance of the intestinal barrier through promotion of tight junction and adherence protein expression (Guerrant *et al*., 2008).

Combined vitamin A and zinc supplementation resulted in improvements in the L:M ratio in young children living in Brazil (Chen *et al*., 2003). In this study, the L:M ratio had an inverse correlation to serum retinol concentrations. The effect of vitamin A supplementation alone is not possible to elucidate from this study design. Vieira *et al*. (2008) examined associations between various biomarkers of vitamin A status and the intestinal integrity of children living in a poor area of Brazil. They found that serum carotenoid concentrations (beta-carotene, beta-cryptoxanthine and lutein) were inversely correlated to L:M ratios. In contrast, serum retinol or serum retinol binding protein concentrations were not significantly related to the L:M ratio. The authors discussed the possible effect of acute-phase serum protein concentrations on serum retinol. They stated that serum carotenoids are not influenced by acute-phase serum proteins which could explain the correlation between serum carotenoids and the L:M ratio but not serum retinol and the L:M ratio.

Vitamin A supplementation has been effective at reducing diarrhea and is associated to reduced infection (reductions in fever, measles, all-cause mortality and a more rapid clinical recovery) in various clinical trials with children from countries such as Ghana, Bangladesh, Brazil and India (Kirkwood *et al*., 1993; Barreto *et al*., 1994; Bhandari *et al*., 1994; Hossain, 1998). It is not certain whether the beneficial effect from vitamin A supplementation is due to correction of deficiency or if additional vitamin has health benefits (Duggan *et al*., 2002). The protective effect of vitamin A against mortality could be through its action as an adjuvant to vaccines (Benn *et al*., 2003). There is a need for evidence demonstrating that vitamin A supplementation results in improved intestinal integrity in populations of children with varying degrees of vitamin A deficiency.

1.1.6.d Probiotics

Probiotics are live microorganisms present in fermented foods that modulate the

intestinal microflora in a manner that improves health (Fuller, 1992). Some of the health benefits from probiotics include enhancing the mucosal immune response, reducing translocation of bacteria, and improving intestinal components such as strengthening tight junctions and promoting mucus secretion (Duggan *et al*., 2002). Probiotics are effective at improving various gastrointestinal conditions including pathogenic infections with rotavirus and *Clostridium dificile*, diarrhea, ulcerative colitis and NEC (Duggan *et al*., 2002).

Due to their protective effect on the intestinal barrier and competition against intestinal pathogens, probiotics may help treat intestinal enteropathy. Egyptian children had significantly improved L:M scores after a 6-week daily supplementation period with yogurt containing *Lactobacillus* (Mohammad *et al*., 2007). Preterm infants fed formula fortified with *Bifidobacter lactis* had significantly lower L:M values compared to control after 30 days (Stratiki *et al*., 2007). Patients with Crohn's disease currently in remission had small improvements in the L:M ratio after 3-month treatment with *Saccharomyces boulardii* compared to control (Garcia-Vilela *et al*., 2008). In a rat model of NEC, supplementation with *Bifidobacteria bifidum* resulted in improved expression of tight junction proteins and reduced expression of IL-6 in the ileum (Khailova *et al*., 2009). The challenge with probiotic treatment is to determine the appropriate dose and micro-organism that will result in the greatest benefit for intestinal enteropathy.

1.1.6.e Summary

All of the above mentioned nutrients could help improve the structure and/or function of the small intestine. However, no single nutrient will cure intestinal enteropathy. Nutrition therapy must be used in conjunction with other strategies. It is likely that different populations of children will require different nutritional therapies depending on their specific nutrition status.

1.2 Giardia intestinalis

1.2.1 Biology

Giardia is the genus name of an expanding number of species of the protozoan parasite that widely inhabits the small intestine of most vertebrates (Thompson, 2002). *Giardia* is found in birds, amphibians, rodents, wild and domestic mammals (Thompson, 2002) and fish (Yang *et al*., 2010). *Giardia* is the most prevalent parasite in humans worldwide (Anderson & Neumann, 2007).

The lifecycle of *Giardia* alternates between the trophozoite and cyst stage (Heresi & Cleary, 1997; Vesy & Peterson, 1999). Outside of its host, it exists as a resistant cyst in both water and land (Heresi & Cleary, 1997; Anderson & Neumann, 2007). Once ingested, exposure to stomach acids initiates the excystation process involving degradation of the cyst wall and the emergence of two trophozoites from every cyst (Erlandsen *et al*., 2002). Each trophozoite has 4 pair of flagella and is able to attach to the enterocytes of the proximal small intestine through motility and via a suction disk located on their ventral surface (Thompson & Monis, 2004). The trophozoite is dependent on the host for fatty acids and it is thought that the localization to the proximal small intestine is intended to access the host's bile (Vesy & Peterson, 1999). *Giardia* reproduce by binary fission during the trophozoite stage (Erlandsen *et al*., 2002). Encystation is thought to initiate from exposure to sufficient concentration of bile and involves synthesis of the cyst wall (Erlandsen *et al*., 2002). Cysts are then excreted in the feces where they can survive in the environment and contribute to further infections if ingested (Thompson, 2002; Heresi & Cleary, 1997). At some point after encystation but before excystation, there is doubling of the genetic material in preparation of eventual cytokinesis as the parasite transforms back into the trophozoite stage (Erlandsen *et al*., 2002).

1.2.2 Definitions

For clarity, the following definitions for each of the listed terms will be used throughout this thesis.

Giardiasis: The state characterized by a person or animal acting as a host or reservoir of the parasite *Giardia* with no specification of presence or absence of symptoms.

Giardia infection: The state characterized by a person or animal with symptomatic giardiasis.

Giardia carriage: The state characterized by a person or animal with asymptomatic giardiasis.

Giardia intestinalis: The accepted species name of the *Giardia* parasite that infects humans and is synonomous with *G. duodenalis* and *G. lamblia.*

1.2.3 Taxonomy

The species name *G. intestinalis* has been used interchangeably with *G. duodenalis* and *G. lamblia* in scientific literature (Thompson, 2002; Buret *et al*., 2002). All three names have arisen due to complications associated with the emergence of a naming system for the *Giardia* species (Van Keulen, 2002). The first attempt to name this parasite was in 1859, the genus *Giardia* was originated in 1882 and it was not until 1952 that a temporary species taxonomy was established by Filice (Van Keulen, 2002). Filice proposed 3 species as determined by morphological characteristics; *G. muris, G. agilis* and *G. duodenalis* (Van Keulen, 2002). Currently, there are 6 accepted *Giardia* species

(Table 1-2) (Adam, 2001). *Giardia intestinalis* has been subdivided into several assemblages based on genetic differences (Table 1-3) (Adam, 2001).

G. intestinalis in the species found in humans (Anderson, 2005). There are several assemblages of *G. intestinalis* (Adam, 2001). Each assemblage has a similar morphology but distinct genetic material (Thompson, 2002). Distinguishing between assemblages therefore requires molecular techniques that can analyze genetic material (Monis *et al*., 2009; Thompson, 2002). Some of the assemblages are limited to a single host. Other assemblages have shared hosts such as assemblage A-1 which is found in human, beaver, cat, lemur, sheep, calf, dog, chinchilla, alpaca, horse, pig, and cow (Adam, 2001). Debate as to whether each assemblage should be designated as a distinct species continues (Monis *et al*., 2009; Van Keulen, 2002).

1.2.4 Zoonosis

Zoonosis is the natural transmission of an infectious disease from vertebrate animals to people, or vice-versa (WHO, 2010). It has been proven through cross-infection studies that some *G. intestinalis* assemblages can be hosted by species other than their natural host (Thompson & Monis, 2004). The frequency of such an exchange occurring outside the laboratory settings is unknown (Thompson & Monis, 2004). This suggests that *Giardia* infection is a potential zoonosis but more evidence is necessary to determine the frequency of zoonotic transmission. Within the *G. intestinalis* species, only assemblage A and B are thought to exhibit zoonosis. Other assemblages have limited and specific hosts (Thompson, 2004).

It has been suggested that humans living in close contact with domestic animals (companion or farm animals) may be at risk of contracting giardiasis from the animal (Thompson, 2002). Giardiasis is common in companion dogs and cats (Bugg *et al*., 1999) and it has been shown in certain cases that dogs and humans

may both harbour the same *G. intestinalis* genotype (Hopkins *et al*., 1997). The prevalence of giardiasis in beef and dairy calves is as high as 100% (O'Handley *et al*., 1999; O'Handley 2002; Ralston *et al*., 2000; Xiao, 1994). Livestock are predominantly carriers of assemblage E but have been reported to occasionally carry assemblage A (O'Handley *et al*., 2000; Appelbee *et al*., 2002). Studies that have compared the position of beef farms to the reported prevalence rates of giardiasis in humans have not found strong associations (Odoi *et al*., 2004). A study in India found that dogs living in close proximity to humans were carriers of *G. intestinalis* Assemblage AII (Traub *et al*., 2005). This situation in is contrast to dogs living predominantly outside the house or in herds who typically would carry *G. intestinalis* Assemblage C/D (Hopkins *et al*., 1997). It appears that carriage of assemblage A by companion dogs is due to human fecal contamination (Traub *et al*., 2005). However, once a companion animal is infected, it serves as a reservoir and source of potentially infective cysts for humans (Traub *et al*., 2005; Anderson & Neumann, 2007). In situations of high prevalence of giardiasis, the host specific strain is thought to have an advantage in colonization within the small intestine over the zoonotic strain (Thompson, 2002; O'Handley *et al*, 2000). If competition for colonization is not high, the zoonotic strain may predominate (Thompson, 2002). Therefore, under certain conditions domestic animals could potentially pass *Giardia* infections onto humans.

Wild animals are mainly carriers of the non-human *G. intestinalis* assemblages. In general, the prevalence of giardiasis among wild animals is much lower than farm animals. Prevalence rates have been estimated at 2-3% in wild animals (Heitman *et al*., 2002). In North America, some studies have found that as high as 50% of wild animals can be carriers of *Giardia*, some of which may be infected with human assemblages (Thompson, 2004; Dixon *et al*., 2002; Measures & Olson, 2002). Wildlife have been determined as contributors to *Giardia* outbreaks associated to contaminated drinking water (Weniger *et al*., 1983). It has been suggested that the cases in which wildlife are determined as the source

of *Giardia* outbreaks, it is possible that the animals were originally infected with human assemblages of *Giardia* from contaminated water due to human wastes (Olson & Buret, 2001; Dixon *et al*., 2002). The human assemblages of *Giardia* can then survive and propagate within the wild animal. The animals act as vehicles of infection amplifying cysts and further contributing to water contamination (Thompson, 2004). If this is true, the original source of giardiasis in certain water outbreaks is humans. This theory is supported by findings from a study in Southern Alberta (Thompson, 2004).

Acceptance of *Giardia* as a zoonosis is still under debate. The taxonomy of *Giardia* spp. needs to be revised utilizing the accumulating genetic information of the different assemblages. Causative evidence of humans being infected with *Giardia* spp. of animal origin is necessary (O'Handley, 2002; Dixon *et al*., 2002).

1.2.5 Etiology

Transmission of *Giardia* is through ingestion of contaminated water or through the fecal-oral route (Thompson, 2004; Ali & Hill, 2003). The fecal-oral route is due to inadequate hygiene in infected people or care givers of infected people (Thompson, 2004; Ali & Hill, 2003). Ingestion of contaminated food is another potential route although it has been stated that this mode of transmission is less common (Ali & Hill, 2003). In North America, transmission of *Giardia* has been associated to swimming pools, daycares, institutions, campers and returned travelers (Heresi & Cleary, 1997; Heresi *et al*., 2000; Yoder & Beach, 2007). Transmission through infected animals is another possibility as discussed in the zoonosis section.

1.2.6 Prevalence

Giardiasis is the most prevalent parasitic infection in humans worldwide

(Andersen & Neumann, 2007). In developed countries, prevalence in selected stool samples is estimated to be between 2-5% (Ortega & Adam, 1997) with higher rates associated to waterborne outbreaks, infant day-care centres, recreational swimming and returned travellers (Heresi & Cleary, 1997; Heresi *et al*., 2000; Yoder & Beach, 2007). In developing countries, stool samples suggest a prevalence of 20-30% (Ortega & Adam, 1997) and high rates of re-infection among children (Saffar *et al*., 2005). It is estimated that in developing countries, 100% of infants under 2-years will become infected (Heresi *et al*., 2000). Accurate global estimates are lacking; quality and quantity of surveillance varies according to country. Prevalence in some countries is limited to accessible population and may reflect daycares, hospital settings, or is specific to either urban or rural communities (Gideon Informatics, 1992). Prevalence of asymptomatic people may be largely underestimated (Yoder & Beach, 2007).

1.2.6.a Europe & North America

Giardia has been identified as the cause of over 40% of waterborne outbreaks associated to protozoan parasites reported in North America and Europe from mainly 1969 to 2001 (Karanis *et al*., 2007). The 132 cases reported during that time period is thought to be a conservative estimate due to limited surveillance, especially in countries other than the United States (Karanis *et al*., 2007).

1.2.6.b Daycare Centers

Daycare centers may have higher rates of *Giardia* compared to the rest of the community. Previous studies have reported prevalence rates of 10% to 57% (Pickering *et al.*, 1984; Ish-Horowicz *et al*., 1989; Janoff *et al*., 1990; Rauch *et al*., 1990; Mendoza *et al*., 2001; Saffar *et al*, 2005). See Table 1.3.

1.2.6.c Developing Countries

Giardia is described as a ubiquitous organism in many developing countries, however, comprehensive continual surveillance is lacking. Most epidemiological data is based on cross-sectional studies conducted in specific communities or institutions. Some of these studies focus on specific age groups or populations (hospitalized, school-age, immuno-compromised, ect.). Generalizations from such studies would not be accurate. Prevalence rates of symptomatic giardiasis from Asia, Africa and Latin America have been reported as 200 000 million cases per year (Thompson, 2002).

1.2.6.4 Guatemala

Recent studies in Guatemala indicate that between 10.9% and 30% of children aged 1 to 15 years from various communities are carriers of *G. intestinalis* (Jensen *et al*., 2009, Cook *et al*., 2009).

1.2.7 Pathogenesis

Typically, *Giardia* does not invade the enterocyte (Faubert, 2000). However, its presence in the small intestine may cause malabsorption of food and nutrients and damage to the enterocytes (Buret *et al*., 2002). The majority (35-70%) of people with giardiasis do not have symptoms (Heresi & Cleary, 1997). When symptoms occur, they mainly include gastro-intestinal distress such as diarrhea, cramping, anorexia, nausea and vomiting (Heresi & Cleary, 1997; Muller & Von Allmen, 2005). The onset of symptoms occurs on average 6 to 15 days after ingestion of cysts in human adults (Vesy & Peterson, 1999). The lowest infective dose is 10 cysts (Vesy & Peterson, 1999). In most cases, the infection is self-clearing (Vesy & Peterson, 1999; Hawrelak, 2003). However, some individuals exhibit chronic giardiasis (Hawrelak, 2003). Factors that are thought to influence the duration of carriage and severity of pathogenesis are the immune status and age of host (Faubert, 2000), malnutrition (Muller & Von Allmen, 2005), the amount of cysts

ingested (Faubert, 2000), and the strain assemblage (Williamson *et al*., 2000; Mank & Homan, 2002).

1.2.7.a Structural Changes to Enterocytes

Symptomatic giardiasis is sometimes associated with damaged enterocytes in the duodenum and jejunum (Heresi & Cleary, 1997). Deletrious effects include villi atrophy, crypt hyperplasia and shortening of the microvilli (Heresi & Cleary, 1997; Koudela & Viovec, 1998; Andersen & Neumann, 2007). This results in a decrease in the absorptive surface area (Buret *et al*., 2002). Treatment for giardiasis in infected animals resulted in increased brush border surface area, increased intestinal disacharidase activity and decreased intraepithelial lymphocytes (O'Handley, 2002). Buret (2005, 2007) concluded that shortening of the microvilli may be more important than villi atrophy in terms of enzyme function and absorptive capacity of enterocytes.

Oberhuber and Stolte (1990) found no difference in villous flattening in 80 subjects diagnosed with giardiasis compared to controls. Most of the subjects had intermediate (41%) to heavy (34%) colonization although clinical symptoms such as diarrhea or malabsorption were not described in the paper. The control subjects were negative for giardiasis but had upper gastrointestinal complaints. Giardiasis may cause alterations in the cytoskeletal protein structure. The proteins thought to be affected include F-actin, alpha-actinin, villin and ezrin (Teoh & Buret, 1999; Teoh *et al*., 2000; Buret *et al*., 2002). These proteins are involved in microvilli structure (Buret *et al*., 2002). Disruption of tight junction proteins has also been associated to giardiasis (Andersen & Neumann, 2007). Potential target proteins include the zona-occludens family, cingulin and claudins (Buret *et al*., 2002). Giardiasis may also cause increased apoptosis of enterocytes (Chin *et al*., 2000).

1.2.7.b Functional Changes to the Small Intestine

In animals, giardiasis has been associated to an increased epithelial permeability (Scott *et al*., 2000; Andersen & Neumann, 2007). The increased epithelial permeability in giardiasis may be a result of disruption of tight junctions, re-arrangement of the enterocyte's cytoskeleton or apoptosis of the enterocytes. A study in Nepali children aged 0-5 years found that *G. intestinalis* infection was associated to increased intestinal permeability (Goto *et al*., 2002). Children with giardiasis (n=8) had a higher L:M ratio compared to non-infected children (n=45, p=0.01) or children with helminth infections (n=73, p=0.03). Similarly, mice infected with *G. muris* had a higher L:M ratio compared to paired sham-treated control mice ($p<0.05$) (Scott 2000 *et al*., 2000). The permeability of the stomach and large intestine was not affected. In contrast, a study on infants from the Gambia found no association between the L:M ratio and giardiasis (Campbell *et al*., 2004). In this prospective cohort study, infants were followed from birth to approximately 15 months of age. During this time, 19 out of 38 infants were diagnosed with giardiasis. A possible explanation for the lack in correlation between giardiasis and L:M ratio is that only 2 of the infants had symptomatic giardiasis and the study did not take into account acute (n=12) vs chronic (n=7) cases. The duration of chronic cases ranged between 6 to 20 weeks.

1.2.7.c Inflammation & the Host Immune Response

Giardiasis has been associated to intestinal inflammation. Campbell *et al*. (2004) found a trend of higher intestinal inflammation (measured by fecal neopterin concentrations) in infants with giardiasis compared to *Giardia*-free infants (20.2 umol/L vs 14.8 umol/L, $p>0.05$). Hanevik *et al*. (2007) reported that inflammation in the duodenum was higher in people with chronic giardiasis compared to recovered controls. In contrast, Eckmann (2003) reported that over 95% of giardiasis cases, even when symptomatic, are non-inflammatory.

Intestinal damage may be due to mediators of the host immune response (Muller & Von Allmen, 2005; Buret, 2007). Calves infected with *Giardia* spp. have increased intraepithelial lymphocytes in the jejunum (Ruest *et al*., 1997). Scott *et al*. (2004) infected mice with *G. muris* and isolated lymphocytes from the superior mesenteric lymph node (SMLN). Whole SMLN, CD8+ or CD4+ enriched lymphocytes were transferred to immunocompetent nu+/nu+ non-infected mice and examined the pathogenesis of the jejunum. They found that most of the detrimental effects such as microvilli shortening, decreased sucrase activity and increased crypt:villi ratio were dependent on CD8+ lymphocytes. Both CD4+ and CD8+ lymphocytes were associated to moderately higher intra-epithelial lymphocyte counts. This data suggests that immune activation caused by giardiasis may contribute to disease pathogenesis. The resulting damage would vary between individuals depending on the individual's immune activation.

1.2.7.d Impact on Nutrition Status

Among infants and children, giardiasis has been associated with deficiencies in serum zinc and iron (Demirci *et al*., 2003) and vitamin A (Quihui-Cota *et al*., 2008). Giardiasis has frequently been linked to impaired anthropometrics in children in developing countries (Loewenson *et al*., 1986; Fraser *et al*., 2000; Muniz-Junqueira & Queiroz, 2002; Simsek *et al*., 2004; Al-Mekhlafi *et al*., 2005; Carvalho-Costa *et al*., 2007). A recent study has suggested that this impairment of growth may also be caused by asymptomatic giardiasis (Prado *et al*., 2005). Other consequences include disaccharidase deficiency and fat, glucose, sodium and water malabsorption (Daniels & Belosevic, 1995; Heresi & Cleary, 1997; Andersen & Neumann, 2007; Buret, 2007).

The impact of giardiasis on growth has been explored since Mata (1978). Debate as to whether the parasite has any negative impact continues. Confounding factors that may mask the negative effect of giardiasis on height and weight

include frequent under-detection of giardiasis (due to sporadic cyst shedding and use of less sensitive methods of detection), co-infection with other pathogens and the lack of assessment of intensity of infection. A detailed discussion is given in Chapter 2.

1.2.8 Diagnosis

Diagnosis of giardiasis is through the confirmation of trophozoites in the small intestine or cysts in the feces. The traditional procedure is the Ova and Parasites (O&P) analysis of a stool sample using a light microscope. Duodenal fluid can be analyzed using a light microscope for detection of trophozoites. Techniques that analyze stool samples include antigen detection tests (Enzyme-Linked Immunosorbent Assay (ELISA) and enzyme immunoassay (EIA), direct immunofluorescence assay (DFA) and deoxyribonucleic acid (DNA) tests. Serum and salivary antibodies specific for *Giardia* can indicate exposure to *Giardia*.

1.2.8.a Microscopy of duodenal samples

Duodenal fluid can be sampled through invasive duodenal aspiration with a naso-duodenal tube or endoscope, or through the non-invasive Entero-Test (string test). In the string test, the patient swallows a gelatin capsule containing a nylon string after fasting for 4-8 hours (Jones, 1986). Before swallowing, part of the string is pulled from the capsule and taped to the patients' outer cheek. The string progresses through the gastrointestinal tract assisted by the weighted capsule. The nylon string will absorb gastrointestinal secretions, tissue and trophozoites. The duodenal fluid squeezed from the string is analyzed in normal saline solution by a light microscope for the presence (motility) of trophozoites (Garcia, 2007). Fluid can be preserved and stained for further analysis (Garcia, 2007).

Examination of dueodenal fluid has occasionally resulted in increased detection of giardiasis when combined with fecal microscopy (Ament, 1972; Rosenthal & Liebman, 1980; Goka *et al*., 1990). In contrast, examination of duodenal fluid had no added value when combined with DFA (Genzyme Virotech) (Wahnschaffe *et al*., 2007).

1.2.8.b Fecal Microscopy

There are 3 steps used in the O&P analysis; direct smear, concentration and permanent stained smear (Garcia, 2007). The direct wet smear should be done on fresh, non-preserved stool. This method is designed to detect trophozoite motility. Concentration of stool is done to increase the probability of detection of parasites cysts (Garcia, 2007). This can be done through flotation and/or sedimentation. Sedimentation is achieved by centrifugation resulting in separation of the parasites (they become the sediment) from the fluid. Sedimentation is not as selective as flotation since all parasites are present in the sediment. In contrast, the flotation method can separate parasites of different mass through the use of solvents of specific gravity. This provides a more pure sample to examine under the microscope. Permanent stained smear is used to confirm the identity of a parasite. The stained sample is viewed under an oil immersion microscope at high magnification to enable detailed analysis of the parasite's morphology. Stool samples can be fresh or preserved. Several different types of stains are available; the optimal choice of stain depends on the compatibility with the agent used in stool preservation and the type of parasite. Quantification of cyst load in stool can be done using a hemocytometer.

1.2.8.c Enzyme Immunoassays

EIA and ELISA are similar methods that detect giardia antigen in stool through the use of specific antibodies (Garcia, 2007; Aziz *et al*., 2001). DFA tests use

fluorescently labeled antibodies specific to *Giardia* cyst (Aziz *et al*., 2001). This method has an advantage over EIA and ELISA because it enables the visualization of whole cysts with a fluorescent microscope (Aziz *et al*., 2001). Numerous studies have shown that ELISA and EIA have a high sensitivity and adequate specificity for the detection of giardiasis 156-161 (Zimmerman and Needham, 1995; Jelinek *et al*., 1996; Rocha, 1999; Maraha & Buiting, 2000; Johnston *et al*., 2003; Srijan *et al*., 2005, Selim *et al*., 2009). Specificity is not as high as the O&P method because the *Giardia* cyst is not visualized under a microscope. Some test kits can simultaneously test for other pathogens but this would not screen samples to the same extent as visual analysis by a trained parasitologist using the microscopy method (Aziz *et al*., 2001). DFA has been used in many studies as a reference standard when assessing the diagnostic potential of other antigen tests (Aziz *et al*., 2001, Zimmerman & Needham, 1995).

1.2.8.d Dipsticks or Cartridge Kits

Several different dipstick/cartridge kits are available (Garcia, 2007). The dipsticks are a rapid, field-friendly tool that indicates presence or absence of *Giardia* antigen in stool samples within 10-15 minutes (Garcia, 2007). The sensitivity of the dipsticks are generally much lower than the sensitivity of the EIAs (Johnston *et al*., 2003; Oster *et al*., 2006; Weitzel *et al*., 2006;). The specificity of some dipstick tests are comparable to that of the EIAs (Johnston *et al*., 2003; Oster *et al*., 2006; Weitzel *et al*., 2006). Dipsticks are not quantitative.

1.2.8.e Polymerase Chain Reaction (PCR) and DNA sequence analysis

PCR amplification of DNA enables identification of *Giardia* genotype, which could be useful in determining the zoonotic potential and virulence of each *Giardia* strain (Wilke & Robertson, 2009). PCR amplification may enable cyst detection from stool samples containing as few as 4 cysts (Miller & Sterling, 2007). The use of PCR could be a useful diagnostic technique considering the irregular shedding of cysts from hosts with giardiasis and the possibility of low

cyst numbers present in feces. PCR and DNA sequence analysis is currently not routinely used for diagnosis of giardiasis in clinics (Hove *et al*., 2009).

1.2.8.f Immuno-Antigen Methods

Anti-*Giardia* antibodies can be measured from serum or saliva samples. Due to the non-invasive nature of *Giardia* the immune response is typically localized to the gastro-intestinal tract (Faubert, 2000). Over time, antibodies may be detected in the serum after transportation through the lymphatic system and into the blood. Secretory IgA which can be found in gastro-intestinal secretions such saliva and fluid from the small intestine may serve as a potential diagnostic tool for giardiasis (Garcia, 2007). Development of salivary anti-*Giardia* antibodies may take a few weeks to occur (Shatla *et al*., 2004). The time delay in antibody production is a major disadvantage for this diagnostic test. Serum antibodies do not distinguish between current and past infection and are currently not used as a method of diagnosis (Gilman *et al*., 1985; Faubert, 2000; Garcia, 2007). Previous exposure to *Giardia* may result in residual concentrations of anti-*Giardia* antibodies in the serum (Hawshkes *et al*., 1994; Miotti *et al*., 1986). A problem with the use of anti-*Giardia* antibodies is the potential lack of specificity and the reported occurrence of cross-reactivity resulting in false-positive diagnosis. People who have presumably never been exposed to *Giardia* have demonstrated low levels of anti-*Giardia* antibodies in serum (Jokipii *et al*., 1988). Serum and saliva from non-infected control subjects can recognize certain *Giardia* antigens (Hasan *et al*., 2002). It has been suggested that some individuals produce antibodies that demonstrate a low-level cross-reactivity to *Giardia* antigens (Jokipii *et al*., 1988, Rodriguez *et al*., 2004). Anti-*Giardia* antibodies may be useful for epidemiological studies to indicate the intensity of exposure to *Giardia* (Miotti *et al*., 1985) or age of first exposure (Gilman *et al*., 1985; Miotti *et al*., 1986) from distinct populations.

1.2.9 Treatment

1.2.9.a Pharmaceutical Treatment

The most commonly used drug for the treatment of giardiasis is metronidazole which is 92% effective when given 2-3 times per day for a period of 5 to 7 days (Gardner & Hill, 2001). Metronidazole has not been approved by the US Food and Drug Administration (Huang & White, 2006). Compliance in children may be compromised due to the drug's bitter taste and gastro-intestinal side effects (Gardner & Hill, 2001). Nitroimidazole derivatives such as tinidazole, ornidazole and secnidazole may only require one dose, which helps to reduce side effects (Gardner & Hill, 2001). However, nausea, anorexia, abdominal pain and dizziness have been reported (Gardner & Hill, 2001). Long-term use of nitroimidazole drugs at high dose has caused mutagenesis in bacteria and carcinogenesis in animals (Erlandsen & Meyer, 1984; Gardner & Hill, 2001). Nitazoxanide is a broad spectrum anti-parasitic drug and was approved in 2003 for treatment of giardiasis in children (Huang & White, 2006). Nitazoxanide has minimal side effects and adequate effectiveness against *G. intestinalis* infection. Nitazoxanide is between 67 and 81% effective at eliminating cyst excretion in children (Ortiz *et al*., 2001; Davila-Gutierrez *et al*., 2002; Diaz *et al*., 2003; Belkind-Valdovinos *et al*., 2004). Due to the potential of side-effects including intestinal discomfort, it has been suggested that nitazoxanide should not be used as a prophylactic anti-parasitic agent in endemic areas (Belkind-Valdovinos *et al*., 2004). Other authors have concluded that the short-term effect of drug treatment, the high probability of reinfection, the potential of side-effects such as intestinal discomfort and the development of drug resistance suggest that prophylactic use of anti-parasitic drugs is not a suitable solution for children living in endemic areas (Saffar *et al*., 2005).

1.2.9.b Innovative Treatment

Wheat germ agglutinin (WGA) is a lectin specific for N-acetyl-D-glucosamine. Ortega-Barria *et al*. (1994) demonstrated that WGA binds to *Giardia* trophozoite and cyst glycoproteins. Binding of WGA inhibited *in vitro* trophozoite attachment to culture tube walls. *In vitro* incubation with WGA resulted in suppression of trophozoite growth/proliferation in a dose dependent manner. Cells were arrested during the preparation for mitosis (G2) and the mitosis (M) phases of the cell cycle. WGA was not toxic to trophozoites; growth resumed once WGA was removed from the cell culture media. *In vivo* infection with *G. muris* was attenuated when mice were given intraesophageal WGA treatment (100μg/day for 2 weeks). The mice given WGA had 50% lower mean number of cysts per g of feces after 5-9 days. Trophozoites colonization in the small intestine was reduced by 30% compared to control. The functional consequence of WGA binding is thought to be interference with trophozoite adhesion to the epithelia followed by impairment of growth. The authors point out that it is unusual for a treatment agent that arrests cells during the G2/M phase to be reversible. WGA binding to cysts may prevent excystation and encystation resulting in reduced trophozoite and cyst loads in the small intestine and feces, respectively.

Grant *et al*. (2001) examined the effect of WGA supplementation to treat human giardiasis. Subjects were given 3 capsules per day, each containing wheat germ (2g) to be consumed for a period of 10 days. The approximate daily intake of WGA was 60mg. All asymptomatic subjects were randomized into treatment or placebo (2g of cornstarch daily). The fecal cyst and trophozoite count (formalin-ether concentration) was significantly lower in the treatment group over the 10-day period compared to control ($p<0.01$, repeated-measures analysis of variance (ANOVA)). Wheat germ treatment containing WGA did not result in eradication of giardiasis in this sample population. The authors suggest that the observed reductions in fecal cyst load could help to reduce transmission of giardiasis particularly in infants and young children.

The results from the WGA studies demonstrate that the specific binding to *Giardia* cysts and trophozoites can reduce *Giardia* encystation, excystation and colonization. WGA does not kill *Giardia* and the effect of reduction of trophozoite growth is dependent on continuous presence of WAG. The reduction of infection intensity could help to reduce transmission and possibly symptomology of giardiasis.

1.3 Gangliosides

1.3.1 Classification & Structure

Gangliosides are a sphingolipid composed of a ceramide with an oligosaccharide chain head group containing at least 1 sialic acid residue and are mainly present in the outer leaflet of the cellular membrane of vertebrate animals (Ledeen, 1984). The ceramide base of the ganglioside associates to lipid rafts in the cellular membrane (Merrill, 2008). The oligosaccharide chain extends outside the outer leaflet of the cellular membrane acting as a receptor or potential binding site (Rueda, 2007). The fatty acid of the ceramide is typically saturated, it may be hydroxylated and can vary between 14 to 30 carbons in length (Vesper *et al.*, 1999). Individual gangliosides vary in oligosaccharide chain length, the number and type of sialic acid residues and the fatty acid found in the ceramide base (Lacomba *et al.*, 2010). This variability results in a large family of diverse molecules. See Figure 1-1.

1.3.2 Nomenclature

The Svennerholm nomenclature system for gangliosides is commonly used (Svennerholm, 1963). This abbreviated naming system for gangliosides consists of 3 characters (2 letters and a number) with a possible subscript letter following the number. The first letter is a capital ‘G’ for gangliosides. The second letter

indicates the number of sialic acid residues present in the ganglioside ('M' = mono; 'D' = di; 'T' = tri, etc). The third character is a number ranging from 1 to 4 indicating the order of migration of the ganglioside on thin-layer chromatography and representative of the number of sugar molecules present in the oligosaccharide chain. The formula is [5 – X = #sugar molecules], 'X' being the third character present in the Svennnerholm naming system. The possible subscript letter following the 3-character abbreviation specifies the biosynthetic pathway utilized in gangliosides formation.

1.3.3 Milk Gangliosides

Milk from every mammal contains gangliosides (Lacomba *et al*., 2010). Isolation and quantification of gangliosides from food sources such as milk is difficult partly due to the variation in ganglioside composition and partly due to interference resulting from the composition of dairy products (high content of fat, ceramides, phospholipids and free fatty acids) (Lacomba *et al*., 2010). During ganglioside extraction, there is a compromise between purity and yield. It is possible that the extraction process results in a selective loss in certain types of gangliosides (polar *versus* non-polar). Current ganglioside quantification methods are based on the amount of lipid-bound sialic acid (LBSA) found in the food under investigation (Rueda, 2007). The relative ganglioside composition of that food source (relative percentage of GD3, GM3, GM1, etc) may or may not be known, and therefore is estimated based on broad assumptions. This estimated ganglioside composition must be used in conjunction with the calculated LBSA amounts in order to determine the total ganglioside mass of the sample (Rueda, 2007). As part of this calculation, the mean molar mass of each type of ganglioside (GD3, GM3, GM1, etc) is based on the best available assumptions for that food type. As a result, studies have produced a wide range in values when estimating ganglioside concentration in milk. The ganglioside composition present in milk differs between species (Pan & Izumi, 2000; Lacomba *et al*.,

2010). The milk ganglioside composition within an individual mother will change over time from colostrum, transition and mature milk (Puente *et al*., 1992; Pan & Izumi, 1999; Rueda, 2007). In addition, the chemical composition of an individual ganglioside is slightly different between species (Rueda *et al*., 1998). Table 1-5 compares human and bovine milk gangliosides.

1.3.3.a Implication of the Differences Between Human and Bovine Milk Gangliosides

The World Health Organization (WHO) recommends exclusive breastfeeding up to the age of 6 months (WHO, 2010). The nutrients present in breast milk, including gangliosides, are therefore the first and only nutrients ingested by infants. The mucosal immune system of the neonate does not fully develop until several years after birth (Lawrence & Pane, 2007). Infants rely on breast milk for protective antimicrobial agents, immune factors and various bioactive compounds including gangliosides to fight off infection (Rueda *et al*., 1998; Lawrence & Pane, 2007; Rueda, 2007). The shift in ganglioside composition of breast milk during the initial period of intestinal development of the neonate suggests that gangliosides may exert a role in tissue development (Park *et al*., 2007; Rueda, 2007). Since ganglioside composition in breast milk is species specific, it is likely that human breast milk gangliosides are best suited for human development and prevention of infection. The efficacy of ganglioside in binding to pathogens has been shown to be species specific. Porcine gangliosides are more effective at binding porcine rotavirus compared to bovine gangliosides (Rolsma *et al*., 1998). This specificity may be due to the types of ganglioside present or from variations in the chemical structure such as the sialic acid residue or the ceramide component. Such variations may alter the receptor-ligand interaction between ganglioside and pathogen and/or signal transduction mediators (Martin *et al*., 2003). The brain is the organ with the highest concentration of ganglioside (Ledeen, 1984). Gangliosides may modulate cognitive function such as learning

and memory (McJarrow *et al*., 2009), placing an even greater importance on ganglioside intake from breast milk (Wang *et al*., 2003). Further research characterizing human-derived ganglioside and the impact on infection and development is needed.

1.3.4 Dietary Sources of Ganglioside

Gangliosides are mainly found in the cellular membrane of vertebrates and are therefore only present in foods derived from animals. Gangliosides are abundant in organ meats, and are particularly high in the brain (Wiegandt & Bucking, 1970; Gasa & Makita, 1980; Reglero *et al*., 1982; Koizumi *et al*., 1988; Lacomba *et al*., 2010). Milk and milk products contain ganglioside (Moore *et al*., 2000; Lacomba *et al*., 2010). Gangliosides are present in the milk fat globule membrane (MFGM). When fresh milk is churned to make butter, the majority of the ganglioside ends up in the buttermilk fraction. Therefore milk products derived from buttermilk are good sources of ganglioside. The consumption of ganglioside-rich foods is variable among cultures due to the variation in consumption popularity of organ meats and buttermilk. Breast milk is likely to be the most consistent source of ganglioside across populations. See Table 1-6 for ganglioside content of foods.

1.3.5 Physiological Effects of Ganglioside in Intestinal Tissue

Dietary ganglioside supplementation can induce a wide range of health benefits due to their structural and functional role in key signaling areas of mammalian cells. Cholesterol, ganglioside and other sphingolipids are concentrated in dynamic regions known as lipid rafts in the cellular plasma membrane (Brown & London, 1998; Simons & Toomre, 2000). Certain proteins (mainly with hydrophobic moeities) have a higher affinity to lipid rafts such as doubly acylated, palmitoylated, cholesterol-linked or glycosylphosphatidylinositol-anchored

proteins (Simons & Toomre, 2000). The presence of protein, particularly transmembrane proteins may help stabilize the lipid raft (Simons & Toomre, 2000). The concept of lipid rafts is that proteins with higher raft affinity will associate with rafts and become exposed to selected enzymes (phosphatases, kinases) that alter protein activity and consequently cellular signalling (Simons & Toomre, 2000). Caveolae are plasma membrane invaginations containing the protein caveolin-1 and may have overlapping structural and functional properties with lipid rafts (Brown & London, 1998). It is possible that sphingolipids, gangliosides and cholesterol are concentrated in caveolae (Brown & London, 1998; Zaas *et al*., 2005). Changing the composition of lipid rafts and caveolae through ganglioside enrichment may modify the activity of lipid raft associated proteins. This in turn can modify the function of cells. For example, in polarized epithelial cells, successful signal transduction by the cholera toxin is dependent on the presence of the ganglioside GM1 in caveolae (Wolf *et al*., 1998).

1.3.5.a Decrease in Pathogen Entry

Lipid raft components serve as receptors to bacteria, toxins and viruses (Abraham *et al*., 2005). Receptor-pathogen ligation is thought to induce endocytosis of the pathogen (Abraham *et al*., 2002). Involvement of lipid rafts in bacteria entry is supported by the finding that the endocytosed bacteria *Pseudomonas* was coated by a membrane containing ganglioside and caveolin proteins (Zaas *et al*., 2005). Ganglioside treatment may interfere with pathogen entry through modulation of lipid raft composition. Rats fed a ganglioside enriched diet have reduced cholesterol and caveolin protein in the lipid raft fraction of intestinal tissue (Park *et al*., 2005). Decreased cholesterol is associated to decreased entry of bacterial toxin (Orlandi & Fishman, 1998) and certain microbial pathogens (Goluszko & Nowicki, 2005). Disruption of caveolae as demonstrated by decreased caveolin protein may inhibit pathogen entry (Anderson *et al*., 1996; Abraham *et al*., 2005). Various types of ganglioside can bind to intestinal pathogens (Table 1-7). This

binding characteristic can be manipulated to help decrease pathogen entry by using dietary gangliosides to act as decoy receptors to intestinal pathogens. Binding of pathogens to ingested ganglioside resulting in excretion into feces could prevent binding of the pathogen to the intestinal cell. Modification of the composition of lipid rafts or sequestering toxins and pathogens through ganglioside supplementation may reduce pathogen entry in the small intestine.

1.3.5.b Decrease in Intestinal Inflammation

Lipid rafts are key regulators of various cell signaling cascades (Simons & Toomre, 2000). T-cell receptor (TCR) signaling is an important example of a lipid raft associated cell signaling cascade (Huse, 2009). Activation of the TCR can lead to the production of inflammatory mediators such as cytokines. Excessive inflammation can damage surrounding cells and tissues. Park *et al*. (2005) has shown that milk ganglioside supplementation results in reduced concentration of inflammatory mediators through modulation of the composition of lipid rafts in the plasma membrane of intestinal cells. Rats fed a ganglioside enriched diet for 2 weeks had reduced cholesterol, caveolin protein, diglyceride (DG) and platelet activating factor (PAF) in intestinal lipid rafts. Disruption of lipid rafts as demonstrated by decreased caveolin protein is associated to decreased activation of inflammatory pathways (Triantafilou *et al*., 2002). PAF is involved in signal transduction events that initiate a cascade of inflammatory pathways that can damage the small intestine (Peplow & Mikhailidis, 1990). In Park *et al*. (2007) rats fed a ganglioside enriched diet for 2 weeks had an attenuated inflammatory response to acute lipopolysaccharide (LPS) exposure. Compared to controls, the ganglioside supplemented group had a higher concentration of an anti-inflammatory cytokine (IL-10) and lower concentrations of pro-inflammatory cytokines (IL-1B and TNF-alpha) and lipid derived inflammatory mediators (PAF, prostaglandin E2 (PGE2) (mucosa tissue only) and leukotriene B4 (LTB4)) in plasma and mucosa tissue. Modulation of the

composition of lipid rafts through gangliosides supplementation can help to control the production of potent local and systemic inflammatory mediators. Gangliosides are effective at preventing tissue damage in an *in vitro* model of necrotizing enterocolitis (Schnable *et al*., 2009). *In vitro* incubation of ganglioside with pediatric intestinal tissue resulted in decreased markers of inflammation and improved tissue integrity. Intestinal tissue was incubated for 10hrs with a low concentration of ganglioside or control, and then challenged with LPS or hypoxic conditions. Tissue incubated with ganglioside had reduced release of lactate dehydrogenase (a marker of intestinal integrity) and reduced nitrite and endothelin-1 concentrations after exposure to LPS, and had reduced concentrations of LTB4 and PGE2 after exposure to LPS and hypoxic conditions. This evidence suggests that ganglioside could help control acute local inflammation and maintain intestinal tissue integrity, at very low concentrations. The role of ganglioside treatment for Inflammatory Bowel Disease (IBD) is currently being explored. Results from an in-progress study in adults with IBD are showing promising effects at attaining disease remission (unpublished data). Subjects in this study consumed a concentrated buttermilk fraction enriched in ganglioside everyday for 8 weeks. To date, 5 subjects have completed the study. All have shown improvements in disease scoring and 4 subjects have attained remission. These findings support the possibility of dietary ganglioside being an effective treatment for intestinal inflammatory conditions.

1.3.5.c Enhanced Development of Adaptive Immunity

Ganglioside provides mucosal protection against pathogens by promoting the development of intestinal adaptive immunity. Research has shown that neonatal mice fed a ganglioside-enriched diet have a higher number of IgA secreting cells and a higher concentration of secretory IgA in the small intestine after 28 days (Vasquez *et al*., 2000). Weaning mice fed a ganglioside enriched diet have earlier and greater development of T-helper type 1 (Th1) and T-helper type 2 (Th2)

cytokine secreting cells in the lamina propria and Peyer's patches of the small intestine (Vasquez *et al*., 2001). These findings suggest that dietary gangliosides may play an important role during development of the intestinal immune system.

1.3.5.d Protection of the Intestinal Barrier

Tight junctions are an integral part of the epithelial barrier. Tight junctions are a group of proteins that bind adjacent enterocytes, regulate paracellular ion and solute flow and maintain enterocyte polarity (Boyle & Finlay, 2005; Viswanathan *et al*., 2009). Damage to tight junctions can result in deregulated movement of antigens or microorganisms including pathogenic and commensal bacteria (Boyle & Finlay, 2005; Garrett *et al*., 2010). This could result in excessive inflammation and immunostimulation causing further damage to the small intestine (Lunn, 2000; Boyle & Finlay, 2005). Ganglioside supplementation can help maintain the intestinal barrier through the protection of tight junctions. Rats supplemented with dietary ganglioside have reduced degradation of the tight junction protein occludin after exposure to LPS (Park *et al*., 2008).

1.3.5.e Prebiotic

In vitro studies have shown that sialic acid promotes the growth of *Bifidobacteria* spp. (Idota *et al*., 1994). Ganglioside contains sialic acid and supplementation can influence the microflora of the small intestine. Supplementation to pre-term infants with a ganglioside-enriched formula for a period of 1-month resulted in significantly higher *Bifidobacteria* after 30 days and lower *E. coli* counts in feces after 7 days (Rueda *et al*,. 1998). It is thought that the sialic acid content of gangliosides promotes growth of *Bifidobacteria.* Probiotic treatment with *Bifidobacteria* spp. has various health benefits (Floch & Montrose, 2005) including reduction in diarrhea in children (Sudarmo *et al*., 2003) and improved

immunity (phagocytosis and cellular immunity) (Arunachalam *et al*., 2000; Chiang *et al*., 2000; Gill *et al*., 2001).

1.3.6 Therapeutic Potential in Giardiasis

Ganglioside can induce pathogen toxicity and intestinal repair of the host. Ganglioside causes in-vitro lysis of *G. intestinalis* trophozoites and enhanced in-vivo resistance to *G. muris* in mice (Suh *et al*., 2004). Mice fed ganglioside had a lower number of trophozoites in the small intestine, delayed onset and duration of infection and a lower cyst count in the feces compared to the control group. *In vitro*, the number of live *G. intestinalis* trophozoites was decreased in a dose-dependent manner when cultured with mixed ganglioside after 48 hours. Ganglioside was effective at very low concentrations.

Ganglioside may sequester and prevent binding of important intestinal pathogens and toxins such as enterotoxigenic and enteropathogenic *Escherichia coli, Vibrio cholerae* and *E. coli* enterotoxins, and Rotavirus (Otnaess *et al*., 1983; Laegreid *et al*., 1987; Newburg, 1999; Idota & Kawakami, 1995; Rolsma *et al*., 1998; Delorme *et al*., 2001). Ganglioside supplementation has great potential for enhancing mucosal immunity and maintaining the intestinal barrier. Ganglioside can be supplemented long-term, providing protection against *Giardia* re-infection. They have a creamy, sweet taste and no side effects. Ganglioside supplementation has potential as an innovative solution for long-term parasite and enteropathogen control in children living in high-risk communities.

1.4 Tables & Figures

Table 1-1: Summary of Global Prevalence and Impaired Growth associated to Intestinal Enteropathy in Children

Reference	Country	Number of Subjects & Ages	Mean L:M ratio*	Growth
Campbell *et al*., 2003	The Gambia	N=53 2-15 months	0.17	L:M was inversely associated to HAZ and WAZ (r=-0.41, p<0.001)
Campbell *et al*., 2004	The Gambia	N=72 2-15 months	0.31	L:M ratio was associated to HAZ (p<0.0001)
Goto *et al*., 2009	Bangladesh	N=222 3-15 months	0.18	L:M ratio was associated to WHZ and WAZ
Lunn *et al*., 1991	The Gambia	N=119 3-15 months	0.38	
Williams *et al*., 2007	The Gambia	N=93 4-10 months	0.26-0.29	No growth comparison data
Goto *et al*., 1999	Guatemala	N=47 (30%) 6-11 months	>0.07	Infants with a WAZ < -1.5 had higher mean L:M ratio (p<0.001)
Sullivan *et al*., 1992	The Gambia	N=20 6-31 months	0.49	No growth comparison data
Campbell *et al*., 2003	The Gambia	N=41 0.5-3 years	0.47-0.73	No relationship between L:M ratio and nutritional status (severity of protein-energy-malnutrition)
Goto *et al*., 2002	Nepal	N=167 0-5 years	0.26	No association between L:M ratio and HAZ
Chen *et al*., 2003	Brazil	N=75 2-97 months	0.29	Improvements in L:M ratio were associated to increases in HAZ
Filteau *et al*., 2001	South Africa	N=149 3 months	0.09-0.11	No growth comparison data
Vieira *et al*., 2008	Brazil	N=47 (48%) 0.5-9 years	>0.09	No growth comparison data
Northrop-Clewes *et al*., 2001	Bangladesh	N=66 2-5 years	0.22-0.25	No growth comparison data

Table 1-2: Summary of *Giardia* species and hosts

Species	Hosts
G. agilis	Amphibians
G. muris	Rodents
G. intestinalis (lamblia or *duodenalis)*	Numerous mammals including humans
G. ardeae	Herons
G. psittaci	Psittacine birds
G. microti	Voles and muskrats

Adapted from Adam, 2001

Table 1-3.: Summary of genotypes of *Giardia Intestinalis*

Genotype Designation	Hosts
A-1	Human, beaver, cat, lemur, sheep, calf, dog, chinchilla, alpaca, horse, pig, cow
A-2	Human, beaver
B	Human, beaver, guinea pig, dog, monkey
C	Dog
D	Dog
E	Cow, sheep, alpaca, goat, pig
F	Cat
G	Rat

Adapted from Adam, 2001

Table 1-4: Prevalence rates of Giardiasis reported from daycare centers

Reference	Age	*Giardia* Prevalence (%)	Stool Sampling Freqency	Setting
Saffar *et al*., 2005	1-5 yrs	10%	1x/week Cross-sectional Study	Iran
Mendoza *et al*., 2001	1-5 yrs	54.6%	3x/week Cross-sectional Study	Cuba
Nunez *et al.,* 1999		20%	3x/week Every 6 months for 18 months	Cuba
Rauch *et al*., 1990	1-24 mths	33%	1x/week for 15 months	Houston, USA
Janoff *et al*., 1990	1-61 mths	21%	1x/week Cross-sectional Study	Thailand
Ish Horowicz *et al*., 1989	3-12 mths 12-24 mths 24-36 mths	40% 23% 57%	1x/month for 12 months	Jerusalem
Polis *et al*., 1986	10-60 mths	35% 20%	1-2 stool samples Cross-sectional Study	Washington, DC USA
Woo *et al*., 1986	2-5 yrs 3-5 yrs	8% 6%	1x/week Cross-sectional Study	Guelph, ON Canada Kitchener, ON Canada
Pickering *et al*., 1984	1-36 mths	33%	1x/week Cross-sectional Study	Houston, USA
Keystone *et al*., 1978	6 weeks to 5 yrs	39% 17%	1x/week Cross-sectional Study	Toronto, ON Canada

yrs=years; mths=months

Table 1-5: Comparison of human and bovine milk gangliosides

	Human Milk Gangliosides					Bovine Milk Gangliosides				
		LBSA mg/L	% GD3	% GM3	% Other		LBSA mg/L	% GD3	% GM3	% GT3
References	*Colostrom (Days 1-5)*					*References*			*Colostrom (Day 1)*	
	1	5.8	71	9	20	4	7.5	70	10	15
	2	2.6	46	6	48	5	3.5	N/A	N/A	N/A
	3	9.8	48	3	49					
	Transition Milk (Days 6-17)								*Transition Milk (Days 5-30)*	
	1	4.5	62	18	20	4	2.3	60	25	3
	2	5.0	37	13	50	5	1.2	N/A	N/A	N/A
	3	9.1	34	27	39					
	Mature Milk (Days 18+)								*Mature Milk (Days 90-180)*	
	1	4.0	20	61	19	4	1.4	65	7	7
	2	1.8	15	44	41	5	0.9	N/A	N/A	N/A
	3	8.0	25	36	39					
	Chemical composition of the Gangliosides									
	Type of Sialic Acid (Wang & Brand-Miller, 2003)					Neu5Ac (100%)		Neu5Ac (73%) Neu5Gc (27%)		
	Type of Fatty Acid in the Ceramide (Bode *et al*., 2004)					SFA: 78-82% MUFA: 14-17% PUFA: 4-5%		SFA: 90-92% MUFA: 6-9% PUFA: 2%		

Adapted from Rueda, 2007; 1. Takamizawa et al., 1986; 2. Rueda et al., 1995; 3. Pan & Izumi, 1999; 4. Puente et al., 1992; 5. Martin et al., 2001

Table 1-6: Ganglioside Content of Food

Quantity of Food Containing 10mg of Ganglioside (g or mL)	Food Item	Reference
700 mL	Human milk	Pan & Izumi, 1999
13 g	Bovine Brain	Wang *et al*., 1998
69 g	Beef Liver	Saito *et al*., 2001
82 g	Buttermilk Powder	†
150 g	Kidney/Spleen	Gasa & Makita, 1980
154 g	Egg Yolk	Li *et al*., 1978
235 g	Chicken Liver	Saito *et al*., 2001
891 mL	Fluid Buttermilk	†

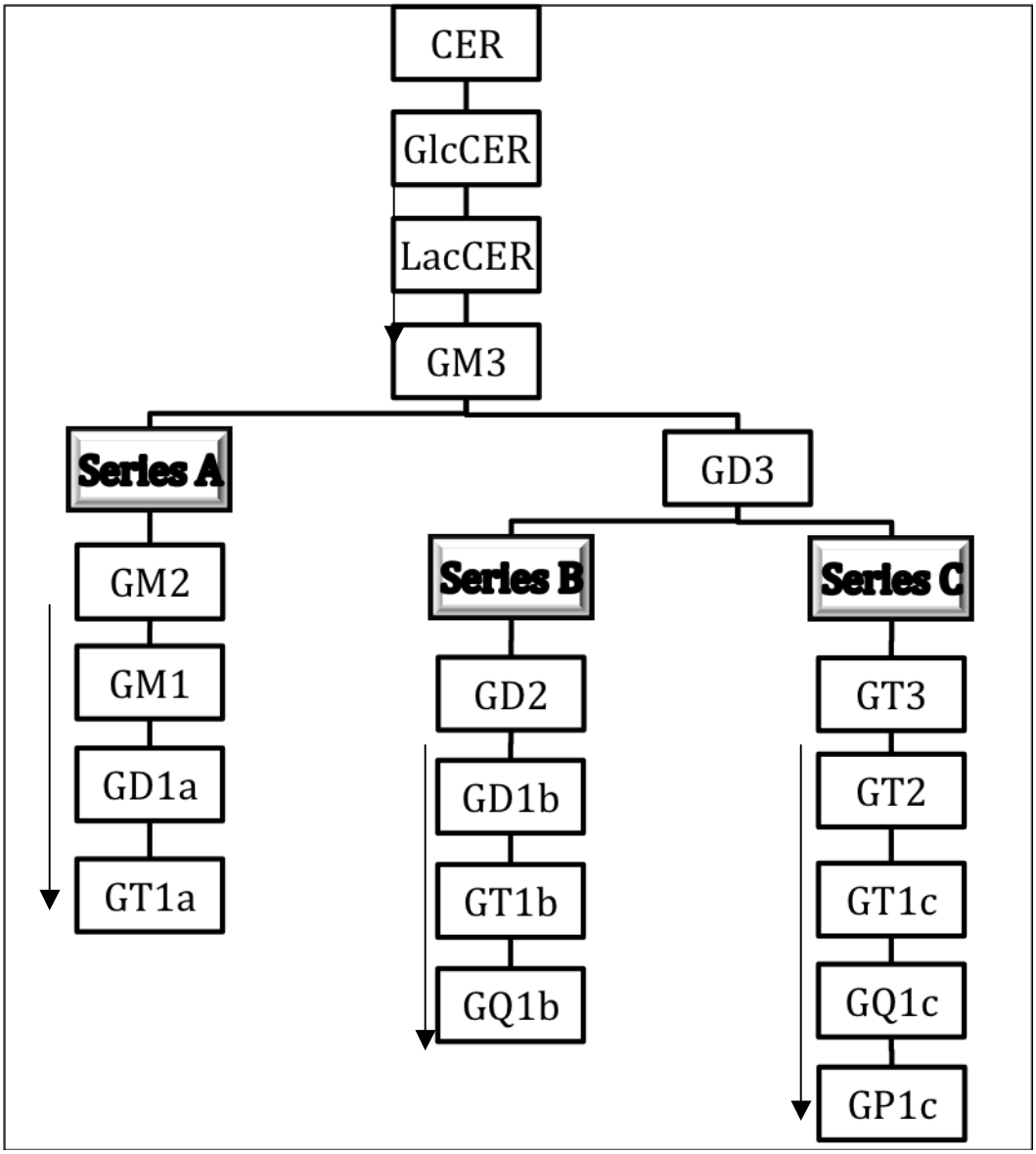
† data not published

Table 1-7: Summary of Ganglioside Pathogen Binding

Ganglioside	Pathogen	Tissue Origen of Gangliosides	Reference
GT1b GD1b	*Clostridium botulinum* neurotoxin C	Rat brain	Tsukamoto *et al*., 2005
GD1a GT1b	Polyoma virus	Purchased from Matreya	Tsai *et al*., 2003
GM1	Simian virus		
GM2 GD1a GM1 GM3	Rotavirus	Bovine small intestine	Delorme *et al*., 2001
GM1	Cholera toxin	Human epithelial intestinal cells	Fantini *et al*., 2000
GM3	Rotavirus	Pig intestine	Rolsma *et al*., 1998
GM1 GM3	ETEC *E. coli* EPEC *E. coli*	Human intestinal cancer cell line	Idota & Kawakami, 1995
GM1	*E. coli* Cholera toxin	Human milk	Laegried & Kolsto Otnaess, 1987
GM1	*E. coli* heat labile toxin Cholera toxin	Rabbit small intestine	Otnaess *et al*., 1983

Abbreviations: ETEC=enterotoxigenic *Escherichia coli*; EPEC=enteropathogenic *E. coli*

Figure 1-1: Biosynthetic pathway of gangliosides; from ceramide to series A, B and C



Adapted from Rueda, 2007.

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CHAPTER 2: PREVALENCE, NEW INFECTION AND SELF-CLEARANCE RATES OF GIARDIASIS IN CHILDREN ATTENDING SEMI-URBAN DAYCARE CENTERS IN GUATEMALA

2.1 Introduction

Giardia intestinalis is an intestinal parasite widely prevalent in children aged less than 5 years (Ish-Horowicz *et al*., 1989; Mahmud *et al*., 2001; Lanata & Mendoza, 2002; Simsek *et al*., 2004; Carvalho-Costa *et al*., 2007; Hollm-Delgado *et al*., 2008). The majority of infections are asymptomatic (Heresi *et al*., 2000). Symptomatic cases mainly include diarrhea, gastro-intestinal pain, anorexia and weight loss (Heresi *et al*., 2000). Children attending daycare centers may be persistent carriers for as long as 12-months (Ish-Horowicz *et al*., 1989). The long-term health consequences of giardiasis have not been fully established. Some studies have found an association between giardiasis and impaired growth (Fraser *et al*., 2000; Muniz-Junqueira & Queiroz, 2002; Sackey *et al*., 2003; Simsek *et al*., 2004; Celiksoz *et al*., 2005; Carvalho-Costa *et al*., 2007). Other studies report no impact on growth (Ish-Horowicz *et al*., 1989; Lunn *et al*., 1999; Campbell *et al*., 2004; Hollm-Delgado *et al*., 2008). Giardiasis has been associated with lower serum zinc, iron (Demirci *et al*., 2003) and vitamin A in children (Quihui-Cota *et al*., 2008; Al-Mekhlafi *et al*., 2010). Some individuals clear *Giardia* without treatment, whereas other individuals need treatment. However, following treatment there is a high rate of re-infection, particularly in daycare settings (Gilman *et al*., 1988; Nunez *et al*., 1999; Saffar *et al*., 2005).

Giardiasis can be diagnosed using fecal tests. Irregular shedding of cysts may result in false negative test results (Garcia, 2007). Collection of multiple fecal samples helps to increase diagnostic rates of giardiasis but this option is not always feasible.

The main objective of this study was to calculate the percentage of children attending daycare with asymptomatic giardiasis, with a 5-week longitudinal component to allow for demonstrating new infection, self-clearance, chronic infection and no infection of giardiasis over that interval. The secondary objective was to evaluate the increase in diagnostic rate as a result of collecting a second fecal sample in a one-week period.

2.2 Methods

2.2.1 Subjects

Three public daycare centers were chosen to participate in this study based on proximity to Quetzaltenango and number of children. All 3 daycares are funded by the *Secretaria de Obras Sociales de la Esposa del Presidente* (SOSEP). Children attending the participating daycare centers were invited to participate in the study. The study was presented to parents and daycare staff at the monthly parent meeting. Parents were given the opportunity to ask questions and discuss the study with the study researchers. Once the parent/legal guardian indicated willingness to participate, the child was screened for inclusion/exclusion criteria by the study researcher.

2.2.2 Study Design

This was a 5-week pilot study to assess prevalence and carriage of *Giardia* in children attending daycare near Quetzaltenango, Guatemala. Two stool samples per week were collected from each subject during week 0 and week 4 (total of 4 samples per subject). Stool samples were analyzed to determine presence or absence of *Giardia.* The parent/legal guardian of any child diagnosed as *Giardia*-positive during week 4 of the study was offered free metronidazole treatment for their child. Treatment was provided by a local doctor.

2.2.3 Inclusion/Exclusion Criteria

All children attending the selected daycare center were invited to participate in the study. Children who were taking medication to treat bacteria, viral or parasitic infections were excluded from the study since medication could affect carriage of *G. intestinalis*.

2.2.4 Stool Collection and Analysis

Collection kits were given to each parent the day before the collection date. Instruction on how to use the kits was provided after written informed consent was obtained. Parents brought back the filled container the following morning and study staff transported the fecal samples to the nearby laboratory *La Democracia*. Samples were refrigerated for up to 48 hours and then were kept frozen at -20 degrees Celsius. The ProSpecT-Giardia-EZ-microplate ELISA method (Remel, Lenexa, Kansas, USA) was used to analyze all fecal samples according to the kits instructions. Samples were kept frozen until analysis.

2.2.5 Data Analysis

The percentage of subjects demonstrating new infection, self-clearance, chronic infection and no infection was determined for the entire study group. New infection rate was calculated as the number of children demonstrating new infection by week 4 divided by the total number of children with no infection in week 0. Self-clearance rate was calculated as the number of children initially infected with *Giardia* who become non-infected by week 4 divided by the number of infected children in week 0. Total prevalence rate of giardiasis was determined for week 0 and week 4 based on the ELISA results. The prevalence rate using only the first stool sample was calculated and compared to the prevalence rate using both stool samples.

2.2.6 Anthropometrics

Height and weight information for each subject was available at the time of the study. Weight was measured using a weight scale and was recorded to the nearest 0.1 kg. Height was measured using a standing measuring board to the nearest 0.5 cm. Data was analyzed using the World Health Organization (WHO) 2006 and the Centers for Disease Control and Prevention (CDC) 2000 child growth standards to calculate the median for weight-for-height (WHZ), height-for-age (HAZ) and weight-for-age (WAZ) (de Onis *et al*., 2006; Kuczmarski *et al*., 2002).

2.3 Results

2.3.1 Subjects

Informed written parental consent was obtained for n= 53 children. A description of the subject population is shown in Table 2-2. The percentage of children with global malnutrition (WAZ <-2 S.D.) ranged between 20.0-26.7% (WHO 2006) or 20.0-46.2% (CDC 2000), stunting (HAZ <-2 S.D.) ranged between 33.3-84.6% (WHO 2006) or 26.7-60% (CDC 2000), and wasting (WHZ <-2 S.D.) ranged between 0.0% (WHO 2006) or 7.7-20.0% (CDC 2000). Of the 53 children enrolled, 48 children provided at least 1 stool sample; 23 children provided all 4 samples and 38 children provided at least one stool sample at both time-points (see Table 2-3). The range of calendar days from the collection of the first fecal sample to be collected and the last specimen obtained was from 31 to 50 days, with a median of 35 days. A description of the 4-stool cohort population is shown in Table 2-4. The percentage of children with global malnutrition (WAZ <-2 S.D.) ranged between 0.0-20.0% (WHO 2006) or 20.0-50.0% (CDC 2000), stunting (HAZ <-2 S.D.) ranged between 30.0-87.5% (WHO 2006) or 30.0-62.5% (CDC 2000), and wasting (WHZ <-2 S.D.) was 0.0% (WHO 2006 and CDC 2000).

2.3.2 Prevalence of Giardiasis

At baseline (week 0), 43.8% of subjects had stool samples testing positive for giardial coproantigen. A similar prevalence rate (44.7%) was found at end of study (week 4). (Table 2-5)

2.3.3 Percentage of Subjects Demonstrating New Infection, Self-Clearance, Chronic Infection and Non-Infection of Giardia

The description of individuals remaining stable with chronic infection or with non-infection with *Giardia* is provided for the large cohort who gave at least 1 stool at each end of the study in Table 2-6, and is shown graphically in Figure 2-1. Focusing in on the group who completed all requisite fecal collections, the 4-stool cohort had a higher percentage of chronic infection and non-infection cases, and a lower percentage of new infection and self-clearance cases compared to analysis of all subjects who provided 2, 3 or 4 stool samples (Table 2-6). The median length of time between first and final stool collection was 5 weeks (median 35 days; range 31 to 50 days) for the whole group (n=38) and 5 weeks (median 35 days; range 32 to 43 days) for the 4-stool cohort (n=23).

2.3.4 Detection of Giardia *Cases Using 1 vs 2 Stool Samples*

Analysis of a second stool sample increased detection of *Giardia* by 11.9% (n=5 additional cases) in week 0 and 8.3% (n=2 additional cases) in week 4 (overall increase in detection was 10.6%). Fewer pairs of stool samples were available for analysis in week 4 compared to week 0 (24 vs 42 pairs of samples). Overall, the use of a second stool sample detected 7 additional cases of giardiasis (Table 2-7).

2.4 Discussion

Among the enrolled subjects, 71.7% provided at least 1 stool sample at both time-points (week 0 and week 4). A much lower percentage (only 43.4%) of enrolled subjects provided the requested 4-stool samples. The largest decrease in collection numbers occurred during week 4, for the duplicate specimen. This was partly due to sporadic daycare closure days surrounding the National (Independence Day) holiday. In addition, there were heavy rains and some flooding during the second collection period resulting in lower daycare attendance rates of children and teachers. The presence of the teacher is an important factor since they are the communication link between researchers and the parents. However, an additional selection factor, perhaps with differential responses to the aforementioned adversities for final collection may be at play. If one carefully compares the mean z-scores in Table 2-2 (all participants) and Table 2-4 (only participants who provided all requisite samples), there is an apparent trend to having better average HAZ and WAZ status among those who complied with full collections and those who did not. A plausible explanation is confounding, with a greater motivation to comply in families with the means to have their children growing better. However, study participation is quite demanding on the daycare staff. Successful stool collection is dependent on staff and parent participation. Reducing stool collection barriers (such as reliance on parents to collect the stool samples) in the future would be one way to eliminate any differential participation within an enrolled cohort and ensure more complete overall compliance.

The WHO definition of malnutrition includes components of inadequate and excess nutrition: undernutrition and overnutrition (WHO, 2011). In terms of undernutrition, malnutrition is the compromised intake, absorption or utilization of nutrients leading to sub-optimal growth and maintenance of body tissue. Stunting (HAZ < -2 SD), wasting (WHZ < -2 SD) and global malnutrition (WAZ < -2 SD) are specific terms used by the WHO and are based on a formula which relies on height, weight and/or age information. These terms reflect severe cases of malnutrition. Therefore, a child may be malnourished without necessarily

being categorized as having stunting, wasting or global malnutrition. The 2010 World Health Statistics (WHO Statistical Information System, 2010) indicate that the percentage of Guatemalan children under 5 yrs with stunting (HAZ < -2 SD) is 54.3% and global malnutrition (WAZ < -2 SD) is 17.7%. Our data indicate slightly higher rates of stunting (64.5%) and global malnutrition (22.6%) among the enrolled children < 60 months (n=31). A possible explanation for the increase in stunting and global malnutrition among our sample population could be the high prevalence rate of giardiasis (~44%), with as high as 34.8% of children having chronic infection lasting at least 5 weeks. In addition, the participating daycare centers in this study are targetted towards children with working mothers. The level of poverty in our sample population may be higher than the national average, potentially accounting for the higher rates of stunting and global malnutrition.

The daycare setup is ideal for facilitation of stool collection and treatment of infection, as well as having been shown in epidemiological studies since the 1980s, to be the setting of greatest giardial transmission (Pickering *et al*., 1984; Polis *et al*., 1986; Ish-Horowicz *et al*., 1989). The prevalence of giardiasis was quite high in the present population sample; almost half of all children had positive stool samples for *Giardia*. This prevalence rate was consistent at both time-points spanning a total time of approximately 5 weeks. Previous studies in daycare centers in this region by the CeSSIAM staff in 2009 have demonstrated high prevalence rates ranging from 10 to 57% (unpublished findings: Montenegro-Bethancourt G & Rios MJ, 2009). However, studies located in the Republic of Guatemala in children aged 1-15 years found lower prevalence rates of giardiasis ranging from 10.9 to 30% (Jensen *et al*., 2009; Cook *et al*., 2009). The higher prevalence of giardiasis found in our study could be due to higher detection rates due to the provision of a second stool sample by most children. Apart from Guatemala, giardiasis has attracted so much attention in institutional settings and daycare, because of prevalence rates from around the world, which

range from 6% to 57% (Keystone *et al*., 1978; Pickering *et al*., 1984; Woo & Paterson, 1986; Polis *et al*., 1986; Ish-Horowicz *et al*., 1989; Janoff *et al*., 1990; Rauch *et al*., 1990; Nunez *et al*., 1999; Mendoza *et al*., 2001; Saffar *et al*., 2005).

Un-detection of giardiasis is common and should be expected due to the well-documented sporadic nature of fecal cyst shedding in people with giardiasis. The findings in the present study are consistent with the literature, in which higher detection of giardiasis occurs when a second sample is provided (Polis *et al*., 1986; Goka *et al*., 1990; Hiatt *et al*., 1995; Jelinek *et al*., 1996; Mank *et al*., 1997; Hanson & Cartwright, 2001). The second stool sample detected an additional 7 cases of giardiasis, increasing the known prevalence by 10.6% (from 36.4% to 47.0%). In this study, each stool collection time-point is described as one week. In reality, the daycare was open Monday thru Friday. Kits were distributed on a Monday, therefore each collection week was limited to 4-days. The results reflect 1 or 2 stool samples collected over 4 days. When comparing the infection status classification data of the 4-stool cohort vs all children, it is evident that the lower rates of new infection and self-clearance are due to improved detection of giardiasis. Therefore some of the children who provided fewer than 4 stool samples may be misclassified as self-clearers or cases of new infection. This suggests that the true prevalence rate of giardiasis in this sample population is likely higher than what was detected. This also suggests that cases of giardiasis are very likely to be chronic and that there is a subset of children who appear to be consistently resistant to infection during a 5-week period.

There is published literature on observed rates of spontaneous self-clearance and new acquisition of giardiasis in settings such as daycare centers. Saffar *et al.* (2005) conducted a clinical trial in which 405 children aged 1-10 yrs with asymptomatic giardiasis were randomized to treatment (Metronidazole) or control (B-complex syrup). A portion (n=49) of the subjects were from day care centers. Children successfully treated (n=174, based on 3 negative stool samples) had a re-

infection rate of 34.5% after a period of 3 months. In the control group, 35.3% (n=71) of children had self-clearance of giardiasis after a period of 3 months. Specific re-infection and self-clearance rates for the daycare group were not separately reported.

Gilman *et al.* (1988) conducted a similar study but in a more impoverished environment in Peru. Children aged under 10 yrs with confirmed giardiasis were treated and followed (n=44) for a minimum of 6 months to evaluate re-infection rate. Almost all (43/44) children were re-infected after 6 months, with the mean time to re-infection being 2.6±1.7 months. All re-infected children (n=43) were then followed for up to 6 more months. Self-clearance (4 continuous weeks of negative stool samples after which the child was no longer followed) occurred within 1 month in 35% (n=15) of the children and within 2 months in an additional 8 children (accumulative total of 53%). A significant percent (28%) of children remained infected for 6-11 months after re-infection. In this study, it is not possible to evaluate the duration of time in which self-cleared children remained without infection.

Bartlett *et al.* (1991) conducted a randomized-controlled trial in infants/toddlers (0-35 months) attending daycare centers (n=31) in Maricopa County, Arizona. Daycare centers were randomized to 1 of 3 intervention groups. The interventions ranged in stringency from treatment and isolation of any diagnosed case of giardiasis (symptomatic and asymptomatic) to treatment and isolation only of symptomatic cases. Monitoring of prevalence rates occurred 4x (single stool sample) over a period of 6 months following initiation of the intervention. Initial *Giardia* prevalence rates ranged between 18-22%. After 6-months of on-going intervention, 6-7% of the original sample population remained infected with no significant differences between any of the 3 intervention groups. Newly enrolled children (screened and treated accordingly upon enrollment) had a *Giardia* prevalence rate of 9-15% at the 6-month follow-up. Despite continuous *Giardia*

surveillance and treatment, almost a third of the initial prevalence rate persisted after 6 months. Children who were newly enrolled or who were originally infected at baseline were more likely to be infected at the 6-month follow-up compared to all other children. Children aged 12-35 months were more likely to have giardiasis throughout the study.

Danciger & Lopez (1975) studied the *Giardia* excretion patterns (cysts and trophozoites) of a group of symptomatic children (n=15) aged 3-7 yrs residing in a Metabolic Unit. The objective was to analyze every stool from each child for the duration of their stay, a period of 33-87 days. Three patterns of cyst excretion were documented; high, low and mixed excretors. The high excretors had on average of over 500 cysts/mg of stool and normally more than 90% of their stool samples tested positive for *Giardia*. The low excretors had between 10-100 cysts/mg of stool and only 20-50% of their stool samples tested positive for *Giardia*. The mixed excretors exhibited phases of high and low excretion and on average, between 60-70 % of their stool samples tested positive for *Giardia*. From these studies, a minority of the *Giardia*-infected population (sometimes as high as one third) becomes and remains persistently infected after successful treatment. Another portion of the population (35-60%) will self-clear within 3 months. However, the data from Danciger & Lopez (1975) suggests that in some studies, the apparent self-clearance rate could actually be under-detection of low or mixed excretors. The one consistent factor among these studies is that children under 10 yrs living in environments endemic for *Giardia*, which to a certain extent includes the daycare setting, consistently have a portion of the population infected. The results from our study indicate that 28.9-34.8% of children are chronic carriers for at least 5 weeks and a slightly higher percentage of children (44.7-47.8%) are consistently not infected with *Giardia*. The remaining percentage (17.4-26.3%), are either chronic infections that are un-detected or they represent cases of new-infection and self-clearance. If we assume that the 3-month rate of self-clearance reported by Saffar *et al*. (2005) over time is linear,

this would represent both a self-clearance and a re-infection rate of ~12%. This is on the order of magnitude of the ~15-20% rates in both directions found in the present study with the most reliable subgroup, those providing all 4 requisite samples.

2.5 Conclusion

Giardiasis is prevalent and chronic in this sample of children attending daycare centers in semi-urban Guatemala. This could have important health consequences. For maximizing validity, *Giardia* prevalence studies should collect at least 2 stool samples per subject in a 4-day period.

2.6 Tables & Figures

Table 2-1: Four Classification Groups of *Giardia* Diagnostic Status According to Stool Sample Results (Positive or Negative)

	Stool Sample Result*	
Infection Group Classification	Week 0	Week 4
New Infection	-	+
Self-Clearance	+	-
Chronic Infection	+	+
No Infection	-	-

*Negative stool sample (-); positive stool sample (+).

Table 2-2: Demographic and Anthropometric Description of Sample of Subjects initially Participating (n=48)

Mean Age (months)	54.1 ± 15.5					
Gender						
Female	N=27					
Male	N=21					
Anthropometrics	WAZ[a]		HAZ[a]		WHZ[a]	
	WHO 2006	*CDC 2000*	*WHO 2006*	*CDC 2000*	*WHO 2006*	*CDC 2000*
Ages						
<35.9 m (n=5)	-1.45±0.71	-*1.84*±0.93	-2.54±0.74	-*2.25*±0.68	-0.05±1.49	-*0.53*±1.50
36-47.9 m (n=13)	-1.08±1.29	-1.23±1.58	-2.16±0.99	-1.83±1.05	0.28±1.14	-0.11±1.33
48-59.9 m (n=13)	-1.73±0.96	-2.01±1.34	-2.65±0.70	-2.36±0.74	-0.18±1.25	-0.54±1.43
≥ 60 m (n=15)	-1.35±0.85	-1.40±0.93	-1.73±1.03	-1.59±1.00	-0.32±0.87[b]	-0.42±0.95
	% Global Malnutrition (WAZ <-2 S.D.)		*% Stunting (HAZ* <-2 S.D.)		*% Wasting (WHZ* <-2 S.D.)	
	WHO 2006	*CDC 2000*	*WHO 2006*	*CDC 2000*	*WHO 2006*	*CDC 2000*
Ages						
<35.9 m (n=5)	*20.0*	*20.0*	*60.0*	*60.0*	*20.0*	*20.0*
36-47.9 m (n=13)	*23.1*	*30.8*	*46.2*	*38.5*	*0.0*	*7.7*
48-59.9 m (n=13)	*23.1*	*46.2*	*84.6*	*69.2*	*7.7*	*7.7*
≥ 60 m (n=15)	*26.7*	*26.7*	*33.3*	*26.7*	*0.0*	*13.3*
< 60 m (n=31)	*22.6*	*35.5*	*64.5*	*54.8*	*6.5*	*9.7*

[a]mean z-score ± S.D.; [b]mean z-scores of BMI for age; WAZ=weight-for-height Z-score, HAZ=height-for-age Z-score; WHZ=weight-for-height Z-score; WHO 2006=World Health Organization 2006 child growth standards; CDC 2000= Centers for Disease Control and Prevention 2000 child growth standards; m=months.

Table 2-3: Stool Sample Collection Rates of Participating (n=48) and Enrolled (n=53) Subjects

	Week 0		Week 4	
	Sample 1/2	Sample 2/2	Sample 1/2	Sample 2/2
Number of Subjects	48	42	38	23
% of Participating Subjects (n=48)	100	87.5	79.2	47.9
% of Enrolled Subjects (n=53)	90.6	79.2	71.7	43.4

Table 2-4: Description of Subject in 4-stool Cohort Population[a], only (n=23)

Age (months)	58.7 ± 13.9					
Gender						
Female	N=13					
Male	N=10					
Anthropometrics	WAZ[b]		HAZ[b]		WHZ[b]	
	WHO 2006	*CDC 2000*	*WHO 2006*	*CDC 2000*	*WHO 2006*	*CDC 2000*
Ages						
<47.9 m (n=5)	-0.76±0.90	-0.84±1.11	-2.21±0.65	-1.88±0.71	0.76±0.88	0.44±1.00
48-59.9 m (n=8)	-1.54±0.76	-1.70±0.89	-2.45±0.59	-2.14±0.61	-0.05±0.87	-0.38±0.95
≥ 60 m n=10)	-1.22±0.87	-1.20±0.88	-1.75±1.22	-1.59±1.14	-0.08±0.76[c]	-0.10±0.65
	% Global Malnutrition (WAZ <-2 S.D.)		*% Stunting (HAZ* <-2 S.D.)		*% Wasting (WHZ* <-2 S.D.)	
	WHO 2006	*CDC 2000*	*WHO 2006*	*CDC 2000*	*WHO 2006*	*CDC 2000*
Ages						
<47.9 m (n=5)	*0.0*	*20.0*	*40.0*	*40.0*	*0.0*	*0.0*
48-59.9 m (n=8)	*12.5*	*50.0*	*87.5*	*62.5*	*0.0*	*0.0*
≥ 60 m (n=10)	*20.0*	*20.0*	*30.0*	*30.0*	*0.0*	*0.0*

[a]These subjects provided the requisite 2 stool samples/week during week 0 and week 4; [b]mean z-score ± S.D.; [c]mean z-scores of body mass index (BMI) for age; WAZ=weight-for-height Z-score, HAZ=height-for-age Z-score; WHZ=weight-for-height Z-score; WHO 2006=World Health Organization 2006 child growth standards; CDC 2000= Centers for Disease Control and Prevention 2000 child growth standards; m=months.

Table 2-5: Global Prevalence Rate of *Giardia* at Baseline (week 0) and End of Study (week 4) Based on ELISA Results

	Prevalence Rate (%)
Global Prevalence at Baseline (n=48)*	43.8
Global Prevalence at End of Study (n=38)*	44.7

*all subjects providing at least 1 stool sample at baseline or end of study were included in this analysis

Table 2-6: Classification of Subjects According to *Giardia* Infection Status during a 5-week Period* Based on ELISA Results

	Subjects with all 4 stool samples (n=23)	Subjects with 1 stool sample at each time-point (total of 2-3 samples) (n=15)	First stool sample of subjects with at least 1 stool sample at each time-point (n=38)
% New Infection (n)	8.7 (2)	26.7 (4)	15.8 (6)
% Self-Clearance (n)	8.7 (2)	13.3 (2)	10.5 (4)
% Chronic Infection (n)	34.8 (8)	20.0 (3)	28.9 (11)
% No Infection (n)	47.8 (11)	40.0 (6)	44.7 (17)
New Infection Rate (%)	15.4	40.0	26.0
Self-Clearance Rate (%)	20.0	40.0	26.7

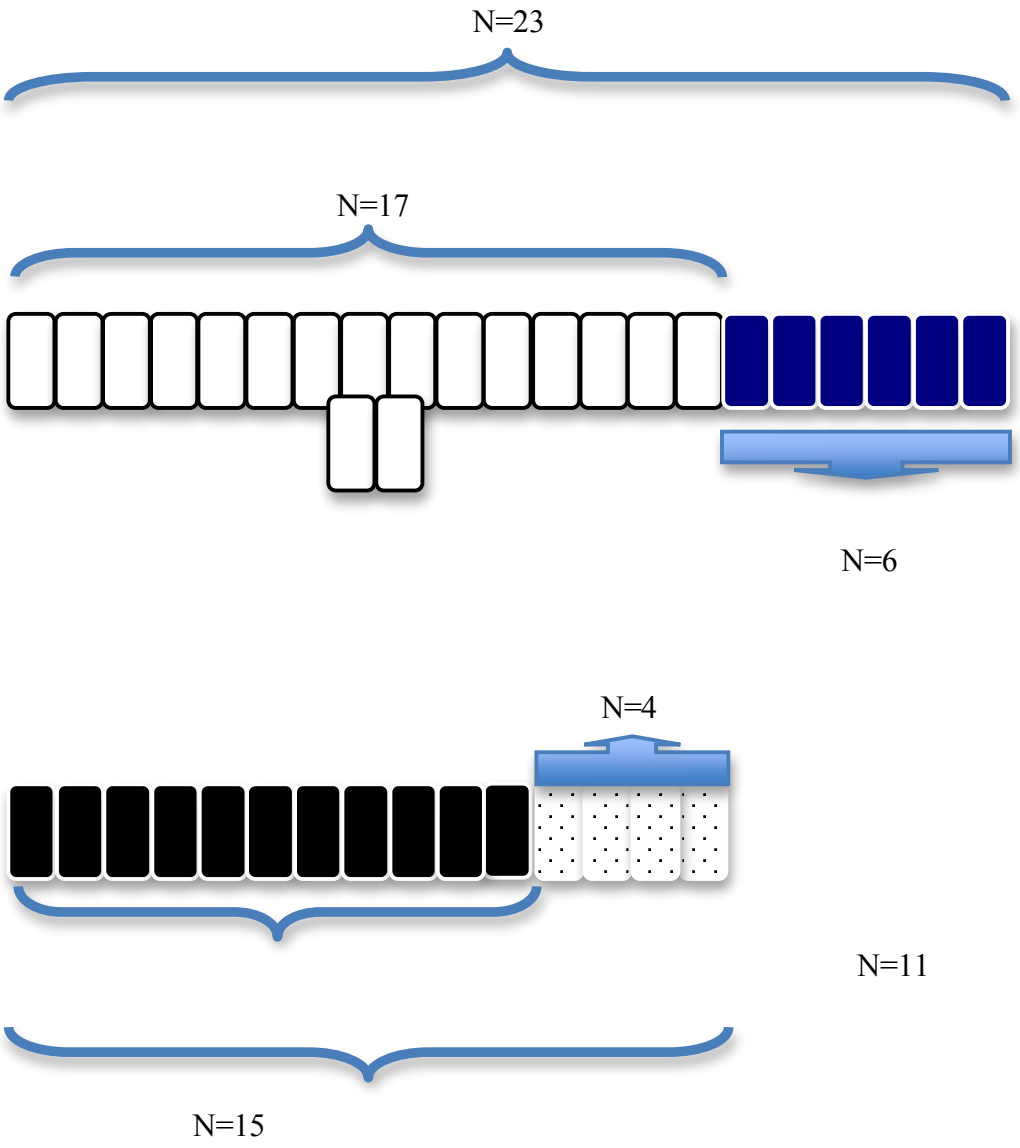
*Mean length of time between collection of first stool sample to final stool sample was 35±3 days.

Table 2-7: Prevalence Rate of *Giardia* using 1 *versus* 2 Stool Samples Based on ELISA Results

	Prevalence Rate % (n)	
	1 Stool Sample	2 Stool Samples
Week 0 (n=42)*	35.7 (15)	47.6 (20)
Week 4 (n=24)*	37.5 (9)	45.8 (11)
Combined (n=66)*	36.4 (24)	47.0 (31)

*only subjects who provided 2 stool samples in a 1-week period were included in this analysis.

Figure 2-1: Schematic representation of *Giardia* diagnostic status at week 0 and week 4 for subjects with at least one fecal analysis at both time points (n=38)



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CHAPTER 3: EXPLORATION OF ASSOCIATIONS AMONG ANTHROPOMETRIC INDICATORS [WEIGHT-FOR-AGE Z-SCORE (WAZ), HEIGHT-FOR-AGE Z-SCORE (HAZ) AND WEIGHT-FOR-HEIGHT Z-SCORE (WHZ)] AND *GIARDIA* INFECTION IN CHILDREN ATTENDING DAYCARE CENTERS IN SEMI-URBAN GUATEMALA

3.1 Introduction

The intestinal parasite *Giardia intestinalis* can damage the small intestine resulting in villi atrophy, crypt hyperplasia, shortening of the microvilli (Heresi & Cleary, 1997; Andersen & Neumann, 2007) and increased intestinal permeability (Scott *et al*., 2000; Goto *et al*., 2002). Giardiasis has been associated with lower serum zinc, iron (Demirci *et al*., 2003) and vitamin A (Quihui-Cota *et al*., 2008; Al-Mekhlafi *et al*., 2010) in children. Giardiasis has been linked to impaired anthropometrics in children in developing countries (Loewenson *et al*., 1986; Fraser *et al*., 2000; Muniz-Junqueira & Queiroz, 2002; Simsek *et al*., 2004; Al-Mekhlafi *et al*., 2005; Carvalho-Costa *et al*., 2007). A recent study has suggested that growth impairment may also be caused by asymptomatic giardiasis (Prado *et al*., 2005). Chronic carriage, self-clearance and re-infection rates could be dependent on nutrition status. In contrast, several studies (cross-sectional and longitudinal) report no impact of giardiasis on WAZ, HAZ and WHZ in children (Ish-Horowicz *et al*., 1989; Lunn *et al*., 1999; Campbell *et al*., 2004; Hollm-Delgado *et al*., 2008). Differences in intensity of infection may have been a confounding factor in previous studies. The main objective of this study was to compare the prevalence rate of giardiasis in well and malnourished children. The second objective was to compare intensity of infection based on ELISA coproantigen values with anthropometric indicators (WAZ, HAZ and WHZ).

3.2 Methods

Detailed description of the subject population, study design, stool collection and analysis, are available in Chapter 2 of this Thesis.

3.2.1 Anthropometrics

Height and weight information for each subject was available at the time of the study. Weight was measured using a weight scale and was recorded to the nearest 0.1 kg. Height was measured using a standing measuring board to the nearest 0.5 cm. Data was analyzed using the World Health Organization (WHO) 2006 and the Centers for Disease Control and Prevention (CDC) 2000 child growth standards to determine the median weight-for-height (WHZ), height-for-age (HAZ) and weight-for-age (WAZ) (Kuczmarski *et al*., 2002; de Onis *et al*., 2006).

3.2.2 Statistical Analysis

Chi-square analysis with above versus below median status for WAZ, HAZ and WHZ was compared to *Giardia* infection status (positive or negative) based on detection of *Giardia* antigen in stool samples. The Spearman correlation value was calculated using the positive ELISA absorbance values and the CDC-2000 z-scores for WAZ, HAZ and WHZ. One ELISA absorbance value per individual was used; if two positive samples were provided then the mean of both samples was used. One-way ANOVA with Kruskal-Wallis for non-parametric data was used to test differences in age (group mean) among the 4 *Giardia* diagnostic status groups. Statistical analysis was performed using GraphPad Prism version 5.03 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

3.3 Results

3.3.1 Anthropometric status of Subjects according to Giardia *Diagnostic Status*

The mean WAZ, HAZ and WHZ z-scores are indicated according to the *Giardia* diagnostic status (new-infection, self-cleared, chronic infection and not-infected) (Table 3-1). Anthropometric data of subjects who submitted all 4 stool samples (n=23) are indicated separately and included in the group analysis (n=37). There

were no statistically significant differences in the age of children according to *Giardia* diagnostic status for the 4-stool cohort (p=0.70) or the group analysis (p=0.45).

3.3.2 Chi-square Analysis for Above vs Below Median WAZ, HAZ and WHZ

The specific 2 x 2 contingency tables for the Chi-square analyses are shown as Tables 3-2 through 3-4. There were no significant associations between *Giardia* infection status (positive or negative) and above vs below median z-scores of WAZ, HAZ or WHZ (p>0.10 for each variable, 2-tailed chi-squared test) (Table 3-5).

3.3.3 Spearman Correlation Analysis for ELISA Coproantigen Intensity vs Z-Score Values for WAZ, HAZ and WHZ

Subjects with a higher ELISA coproantigen intensity tended to have lower WAZ z-scores (r = -0.32, 1-tailed p=0.08). The 1-tailed test was performed after the general consistency of a negative coefficient correlation, i.e. an inverse association between giardial intensity and growth was shown across the board for all three indicators. All other correlations were non-significant (1-tailed p>0.10) (Table 3-6).

3.4 Discussion

The results from previous studies examining the association between anthropometrics and *Giardia* infections in children have been inconsistent. Evaluation of this topic can be dated to the pioneering longitudinal study of growth and infection in the village of Santa María Cauqué, 175 km to the east of Quetzaltenango along the PanAmerican Highway (Mata, 1978), and the secondary analysis of the data by Farthing *et al*. (1986). These researchers in Guatemala

found *Giardia* infection was associated with lower weight velocity (kg/year) between ages 1-2 yrs (p=0.03). Between the ages of 1-2 yrs, *Giardia* episodes lasting longer than 2 weeks tended to be associated with lower weight and height (cm/yr) velocity (not significant). Height velocity was negatively affected in children with 3 *Giardia* episodes lasting at least 2 weeks in duration or when 1-3 *Giardia* episodes were associated with diarrhea ($p<0.05$). During this age period, 22 children had *Giardia* episodes concomitant with other enteropathogens and 12 children had *Giardia*-only episodes. Despite the higher prevalence of diarrhea among the group with mixed infections, there were no differences in height or weight velocities between the 2 groups of children. This suggests that part of the negative impact on growth is likely due to *Giardia*. On average, each subject experienced 7.9 episodes of diarrhea per year. Only 1 *Giardia* episode per year was associated with diarrhea, accounting for approximately 13% of yearly diarrhea. No associations between *Giardia* infection and compromised growth were found in subjects during the ages of 2-3 yrs because there were very low numbers of non-infected subjects (n=3) during this age period. *Giardia* infection had no impact on growth during the first year of life. Cook *et al*. (2009), also working in Guatemala found that children aged 5-15 yrs with increasing global malnutrition (WAZ assessed by WHO 2006 child growth standards) had a higher risk of *Giardia* infection (p=0.0009). Children with moderate global malnutrition (WAZ= 60-74.9% of mean WHO 2006) and severe global malnutrition (WAZ ≤ 59.9% of mean WHO 2006) had an odds ratio (OR) of 1.5 and 4.2 for *Giardia* infection. *Giardia* diagnosis was based on a single fecal sample. Children were not followed longitudinally, however, annual stool collection was conducted in the same region over a period of 4 years.

Sackey *et al*. (2003) found that children (0.2-14 yrs) in Ecuador with stunting (HAZ < -2 S.D. median, National Center for Health Statistics (NCHS) growth reference curves) had an increased risk of *Giardia* infection (OR=2.16, p=0.01). There were no significant associations between *Giardia* infection and global or

acute malnutrition (WAZ or WHZ < -2 S.D. median NCHS). The prevalence of stunting in the Ecuadorian sample population (n=244) was higher (40%) than global malnutrition (21.5%) or acute malnutrition (4.1%). The authors acknowledge that the diagnostic technique (wet smear with staining and analysis by light microscope) was likely less sensitive than other methods. To compensate for this limitation they collected 3 consecutive stool samples from each subject. Fraser (2000), working with Israeli Bedouin groups, found that giardiasis (acute and chronic, n=206) during the first 2 years of life is associated to slightly lower mean z-scores (NCHS) for WAZ (p=0.03) and WHZ (p=0.02) as compared to 24 control toddlers without giardiasis. One of the objectives of this study was to assess the impact of the accumulative duration of *Giardia* infection on WAZ, HAZ and WHZ. No significant associations were found (p>0.05). The authors discuss the potential under-detection of *Giardia* infections in this study due to the limitations of the microscopic analysis (wet smear analysis on 2 consecutive stool samples/month preserved in phenol-alcohol-formaldehyde). This could explain why they found an association between WAZ and WHZ of children presenting at least one *Giardia* infection but no associations with duration of carriage.
In a longitudinal study conducted in Peru, WAZ, HAZ or WHZ z-scores (WHO 2006) were stratified and compared to prevalence and incidence of *Giardia* infections using chi-square test for trends (Hollm-Delgado *et al*., 2008). No significant associations were found when adjusted for age, gender, diarrhea, water and sanitation, maternal height, and per capita household income. In the single-variable analysis, there was a trend of decreased relative risk (RR) of *Giardia* infection with increased WAZ (RR=0.85, 0.76-0.95) and HAZ (RR=0.73; 0.65-0.82) but not WHZ (RR=1.00, 0.91-1.11). The 2-month period following a subject's first episode of giardiasis (first positive stool sample) was not associated with decreased growth (cm/yr or kg/yr) compared to non-infected children, even when stratified by age. In a subset of the sample population (n=108), the long-term prevalence of *Giardia* (proportion of *Giardia*-positive weeks) between the ages of 6-24 months was not associated to mean height gain in the linear

regression model (p=0.981). In this study, children were followed from birth to 3 yrs. A single fecal sample was collected every week and analyzed using light microscopy (direct wet-mount and formalin ether concentrated stools).

The lack of association between above vs below median WAZ, HAZ and WHZ and *Giardia* infection status in the present cross-sectional study in the Guatemalan highlands is consistent with the results from the longitudinal study by Hollm-Delgado *et al*., (2008). However, Hollm-Delgado *et al*., (2008) focused on children from birth to 3 yrs; in contrast our study population was older (98% of subjects were aged 2-7 years, mean age was 4.5 yrs). The study by Farthing *et al*., (1986) and Hollm-Delgado *et al*., (2008) had somewhat similar study designs and yet contrasting results. The data from each study was analyzed using different statistical modelling. Farthing *et al*., (1986) categorized children as *Giardia*-positive or *Giardia*-negative during 3 different age periods (0-51 weeks, 52-103 weeks and 104-155 weeks) and compared the mean growth velocities (height and weight) of the 2 groups. Only growth during the second year was significantly impacted by *Giardia* infection. Grouping children based on any *Giardia* infection over a period of approximately 1 year helps to minimize problems associated to under-detection. Both studies relied on analysis of a single-fecal sample/week which could under-estimate weekly *Giardia* prevalence rates by 11-27% (Goka *et al*., 1990; Hiatt *et al*., 1995; Mank *et al*., 1997; Hanson & Cartwright, 2001). The lack of categorization of each week as *Giardia*-negative or *Giardia*-positive in order to assess the prevalence of *Giardia* could explain the lack of significant associations between infection and growth/anthropometrics found in Hollm-Delgado *et al*., (2008).

It is possible that changes in micronutrient status may show more consistent results as opposed to anthropometrics. The status of iron, vitamin A and zinc could be a more sensitive and specific biomarker for the negative impact of giardiasis on malnutrition. Similarly, measures of intestinal function such as the

lactulose:mannitol test could be used to determine the prevalence of intestinal damage associated to chronic asymptomatic giardiasis. Goto *et al*., (2009) found a trend of improved L:M values in children treated for *Giardia* (scenidazole) compared to control (post-hoc analysis p=0.03). No trend of improved WAZ, HAZ or WHZ was found.

A limitation of the previously discussed studies is their common reliance on light microscopy for detection of *Giardia*. ELISA is a much more sensitive detection method for *Giardia*. This could explain why a higher proportion (67%) of *Giardia* infections in our study were chronic (lasting the duration of the study period, mean of 35±3 days) compared to Hollm-Delgado *et al*. (2008) (31% lasting ≥5 weeks). Under-detection of giardiasis could mask the impact on growth particularly when the prevalence is based on a single stool sample per week analyzed by microscopy.

Handoussa *et al*., (2005) found certain associations between intensity of infection (ELISA OD, Novum Diagnostica) and *Giardia*-associated symptoms. There was a positive correlation between ELISA OD values and symptoms such as severity of diarrhea, nausea, colic, anorexia, weight loss and fatigue. No correlation was found between ELISA OD and the method used to determine cyst count. No other study has looked at the correlation between intensity of *Giardia* infection and anthropometric parameters. In contrast, other intestinal parasites such as Ascaris are often grouped according to infection intensity, using fecal egg counts as the indicator of the number of worms in the intestine (Al-Mekhlafi *et al*., 2010). The results from our present study in Guatemala suggest a trend of greater intensity of *Giardia* infection and lower WAZ. Giardial coproantigen may be reflective of the quantity of colonized *Giardia* in the small intestine. Colonization of *Giardia* has been associated to decreased intestinal absorptive area, increased intestinal permeability, higher nutrient malabsorption and lower markers of serum micronutrient concentrations (iron, zinc and vitamin A) (Scott *et al*., 2000; Buret

et al., 2002; Goto *et al*., 2002; Demirci *et al*., 2003; Andersen & Neumann, 2007; Buret, 2007; Quihui-Cota *et al*., 2008; Al-Mekhlafi *et al*., 2010). Intensity of infection is a potential confounder should be considered in future studies that assess associations between giardiasis and anthropometric values.

3.5 Conclusion

The ages between 3-5 yrs appear to have the highest prevalence of giardiasis, with a substantial proportion of cases lasting at least 5 weeks. To clarify inconsistencies regarding the impact of *Giardia* infection on growth, the longitudinal assessment of height and weight should be compared to *Giardia* infection status using sensitive *Giardia* diagnostic methods that can also measure intensity of infection. Mores studies investigating the impact of giardiasis on micronutrient concentrations (iron, Vitamin A and zinc) and intestinal function could help to determine the parasite's impact on nutrition.

Giardiasis in Guatemala has consistently shown a negative impact on growth in children. Remaining to be thoroughly investigated are: 1) whether this trend is due to differences in research study design or analytical approach, as compared to settings in which a negative association has not been shown, or 2) whether this results in fact from actual geographic/environmental differences.

3.6 Tables

Table 3-1: Anthropometric status of Subjects according to *Giardia* Diagnostic Status

Giardia Diagnostic Status		Subjects with all 4 stool samples (n=23)			
Anthropometric Indices*	n	Age** (months)	HAZ	WAZ	WHZ
New Infection	2	49±8	-1.67±0.19	-1.11±1.25	-0.05±1.48
Self-Cleared	2	57±9	-1.49±1.94	-0.95±1.91	0.11±0.96
Chronic Infection	8	59±10	-1.64±1.06	-0.95±1.03	0.22±0.92
Not Infected	11	61±18	-2.08±0.69	-1.64±0.69	-0.34±0.75
		Subjects with at least 1 stool sample at each time-point (n=37)			
New Infection	6	48±12	-2.05±0.86	-1.93±1.41	-0.84±1.25
Self-Cleared	4	61±13	-2.18±1.40	-2.32±2.53	-1.05±2.35
Chronic Infection	11	57±10	-1.85±1.19	-1.07±1.11	0.26±0.83
Not Infected	16	58±20	-1.98±0.69	-1.79±0.79	-0.67±1.04

*CDC-2000 z-scores, group mean ± S.D.; **mean±S.D., differences between groups were non-significant for the 4-stool cohort (p=0.45) and subjects with at least 1 stool sample (p=0.70) using one-way ANOVA and Kruskal-Wallis test for non-parametric data.

Table 3-2: Chi-square 2x2 Contingency Table for above vs below median WAZ (CDC 2000)

	Giardia-Positive	*Giardia*-Negative	Totals
Above Median WAZ	12	12	24
Below Median WAZ	9	13	22
Totals	21	25	46

Table 3-3: Chi-square 2x2 Contingency Table for above vs below median HAZ (CDC 2000)

	Giardia-Positive	*Giardia*-Negative	Totals
Above Median HAZ	10	14	24
Below Median HAZ	11	11	22
Totals	21	25	46

Table 3-4: Chi-square 2x2 Contingency Table for above vs below median WHZ (CDC 2000)

	Giardia-Positive	*Giardia*-Negative	Totals
Above Median WHZ	13	10	23
Below Median WHZ	8	15	23
Totals	21	25	46

Table 3-5: Summary of Chi-square Analysis for above vs below median WAZ, HAZ and WHZ

Anthropometric Indicator (CDC 2000)	Chi-square (2-tailed, alpha=0.05)
WAZ	X^2=0.382 P=0.5364 (NS)
HAZ	X^2=0.321 P=0.5708 (NS)
WHZ	X^2=2.190 P=0.1389 (NS)

NS=non-significant

Table 3-6: Spearman Correlation Analysis for Giardial Coproantigen Intensity vs z-score values for WAZ, HAZ and WHZ

Anthropometric Indicators (CDC 2000 z-scores)	Spearman Correlation (r) with ELISA coproantigen absorbance values	1-tailed p-value (alpha=0.05)	2-tailed p-value (alpha=0.05)
WAZ	-0.32	0.08*	0.16
HAZ	-0.26	0.12	0.25
WHZ	-0.084	0.36	0.72

*trend of significance

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CHAPTER 4: COMPARISON OF SENSITIVITY AND SPECIFICITY OF 3 FECAL DIAGNOSTIC METHODS FOR GIARDIASIS: [PROSPECT-GIARDIA-EZ-MICROPLATE ASSAY; RIDAQUICK GIARDIA DIPSTICKS VERSUS MICROSCOPIC ANALYSIS]

4.1 Introduction

Giardia intestinalis is the most prevalent protozoan parasite in humans worldwide (Andersen & Neumann, 2007). Giardiasis can be diagnosed using several different fecal tests such as direct light microscopy, Enzyme-Linked Immunosorbant Assay (ELISA) and rapid dipsticks. Microscopic analysis detects whole cysts; ELISA and dipsticks detect giardial coproantigen. The range in sensitivity among the different tests is large. Microscopy is more subjective, but has the advantage of being quantitative. ELISA values may also be used in a quantitative manner if one accepts the assumption that the absorbance reading of a sample is proportional to the actual concentration of giardial coproantigen in the sample. Currently, ELISA results are reported qualitatively (positive or negative); the quantitative optical density (OD) readings are not used to describe intensity of infection.

The objective of this study was to compare the agreement among microscopy, ELISA and a rapid dipstick test in the diagnosis of giardiasis in feces. The relative sensitivity and specificity between pairs of methods was evaluated. The secondary objective was to compare intensity of infection (cyst/gram) determined by microscopy vs the OD readings of positive ELISA samples.

4.2 Methods

4.2.1 Stool Sample Collection & Storage

Stool samples were collected from 3 different daycare centers as part of a larger study. Stool samples were refrigerated for up to 48 hrs in order to conduct the

microscopy analysis. After 48 hrs, stool samples were frozen at -20 degrees Celsius and analyzed within 6 weeks.

4.2.2 Microscopic Analysis for Giardia Cysts in Stool Samples

The sucrose gradient concentration method was used (Roberts-Thomson *et al*., 1976). Fresh stool sample (0.5g) was mixed with 15mL of distilled water to form a solution. The solution was filtered using a metal filter. The filtered stool solution was dispersed equally among 6 centrifuge tubes each containing 2.5mL of 1 M sucrose. Tubes were spun for 15 min at 1000 x g. 3 layers appeared; the top layer was gently removed and discarded using a clipped transport pipette leaving approximately 1cm above the middle layer interface. The remaining top layer and the entire middle layer was collected and emptied into a single 15mL collection tube using a clipped pipette and a gentle suck and rotating method. The collection tube was then topped up completely with distilled water and spun for 10 min at 1400 x g. The supernatant was decanted off and pellet was re-diluted with distilled water to a final recorded volume of 1.0-2.5mL. 2 drops of Lugol's dye was added to each sample. The number of cysts were quantified using a hemocytometer (22mm x 22mm coverslips, thickness #1) and a bright field microscope with high (40x) power objective (Daniels & Belosevic, 1995).

4.2.3 ELISA Analysis of Giardial Coproantigen

Stool samples were kept frozen, a period of from 4 to 42 days, for analysis. The ProSpecT-Giardia-EZ-microplate assay was used to analyze all fecal samples according to the kit instructions. Absorbance values were read using the co-analyzer 303+ with filters of 450 and 630 nm. Samples were read in singlicate.

4.2.4 Dipstick Analysis of Giardial Coproantigen

Samples were kept frozen, a period of from 4 to 42 days, for analysis. The RidaQuick Giardia dipstick test was used on the first fecal sample per subject given per week. The RidaQuick *Giardia* dipstick test uses a package of 25

individual strips. A strip is placed into a test tube containing a specified amount of fecal material (fresh or frozen) that has been mixed with the provided buffer. The test is read as positive or negative (with a control indicator on the dipstick) within 5 minutes.

4.2.5 Selection of Samples for Three-Way Comparison

In total, 151 stool samples were collected from n=48 subjects. All samples were analyzed by ELISA but only a subset of samples were analyzed by microscope (n=75) due to restricted use of only fresh samples. Dipstick analysis (n=99) was done on the first sample from each subject per week to mimic and evaluate the use of dipsticks as a screening tool for giardiasis, and secondly to match all microscope-analyzed samples to enable 3-way comparison.

4.2.6 Data Analysis

Agreement between all 3 diagnostic tests is calculated based on the number of samples in which all 3 tests were unanimously negative or positive, divided by the total number of samples. The sensitivity and specificity of the dipstick and microscopy test are calculated using ELISA as the gold standard.

Sensitivity: $\frac{\text{\# of true positive stools by method X}}{\text{\# stools positive by ELISA}}$

Specificity: $\frac{\text{\# of true negative stools by method X}}{\text{\# of true negative stools by ELISA}}$

Positive Predictive Value (PPV): $\frac{\text{\# of true positive stools by method X}}{\text{[\# of true positive stools by X + \# of false positive stools by method X]}}$

Negative Predictive Value (NPV): $\frac{\text{\# of true negative stools by method X}}{\text{[\# of true negative stools by method X + \# of false negative stools by method X]}}$

4.3 Results

4.3.1 Stool Sample Agreement Among 3 Diagnostic Methods

Agreement among the 75 samples evaluated by all 3 methods was 74.7% (Table 4-2). The highest level of agreement was between the microscope and dipstick analysis (89.3%). The discrepancy in results was due to a false positive sample by the dipstick and 2 false negative samples by microscopic analysis.

4.3.2 Sensitivity, Specificity, and Positive and Negative Predictive Values of the Dipstick Method and Microscopy Relative to ELISA

In general, the microscopic analysis had a slightly higher specificity and PPV (Table 3.6). The dipstick method had a higher sensitivity and NPV. In this set of samples, the sensitivity, specificity, PPV and NPV of the dipstick method deteriorated when more samples were analyzed with 99 samples in the binary comparison with dipstick, as compared to the 75 samples in the 3-way comparison.

4.3.3 Intensity of Infection Comparison Between Microscopy and ELISA

The absorbance values from the ELISA assay range from 0.0 to a maximum of 3.0. Values ≥ 0.05 are considered positive. *Giardia* cysts in stool samples were quantified using a hemocytometer in the microscopic analysis. Comparison of the cyst count (positive samples only, n=15) vs ELISA absorbance values indicates that all of the positive samples detected by microscope had high ELISA absorbance values (>1.7) (Table 4-8). However, the Spearman correlation, using scaling values as proxies for intensity of infection between cyst count and absorbance reading, was non-significant (p=0.26). Alternatively, not all of the samples with high ELISA absorbance values (>1.7) were detected by microscopic analysis (Table 4-7).

4.4 Discussion

Both the microscope and dipstick methods demonstrated low sensitivities but good specificity for the diagnosis of giardiasis in stool samples. In comparison, the ELISA method detected almost twice as many cases of giardiasis.

A disadvantage of the microscope method is that it takes considerably more time for analysis (in this particular situation approximately 10 samples could be analyzed per day) and requires stool samples to be fresh or analyzed within 48 hrs if refrigerated. In comparison, the dipstick method is simple utilizing minimal laboratory equipment, stool samples can be fresh or previously frozen and a batch of 35 samples can be completed within 2 hrs. The ELISA method stands out as the gold standard method in its superior sensitivity, its ease of use particularly if an ELISA reader is available and it is comparable in speed to the dipstick method in that 94 samples can be analyzed in about 6 hrs. ELISA can also be used on fresh or previously frozen stool samples.

As discussed in other studies (Jelinek *et al*., 1996; Vidal & Catapani, 2005; Weitzel *et al*., 2006), the main advantage of the microscope method is the potential to diagnose other parasite infections assuming the analysis is completed by a trained parasitologist.

In this study, the sucrose concentration method was used to selectively concentrate *Giardia* cysts prior to microscopic analysis. Previous studies have generally used the Ovum and Parasite (O&P) series of microscopic analysis. There are 3 steps used in the O&P analysis; direct smear, concentration and permanent stained smear (Garcia, 2007). Some laboratories may choose to omit the direct smear method if preservatives are added to their stool samples (Garcia, 2007). The sensitivity of microscopy using only a wet smear on fresh stool was reported to be 65% compared to ProSpecT ELISA (Oster *et al*., 2006). The sensitivity of microscopy using concentrated stools was 57.2- 75% (Srijan *et al*., 2005; Oster *et al*., 2006). The O&P method resulted in a sensitivity of 66.7% for a single stool sample and 93.3% when a second stool sample from the same patient was examined (Hanson & Cartwright, 2001). In summary, the reported sensitivity of different microscopy methods for single stool analysis has ranged between 57.2-75%. The sensitivity of microscopy from our study is slightly lower at 53.6%.

RidaQuick dipsticks have a reported sensitivity of 80% and specificity of 99.4% (n=220) (Weitzel *et al*., 2006). In this study a sample was determined to be positive for *Giardia* if one of 3 conditions were fulfilled; a) positive result from the O&P and direct fluorescent antibody (DFA) methods, b) a positive result from DFA and at least one enzyme immuno-assay (EIA) method or c) a positive result from 4/5 EIA tests. The sensitivity reported in our study is much lower (50-60.7%) with comparable specificity (96.8-97.9%).

An explanation for the lower sensitivities (microscopy and dipstick) found in our study could be that a large proportion of subjects had lower fecal cysts counts and coproantigen concentrations than was the situation in the comparative studies. Some studies have found that asymptomatic carriers have lower cyst counts (Soliman *et al*., 1998) and coproantigen levels compared to symptomatic cases (Rosoff *et al*., 1989). A substantial portion (37%) of the positive stool samples by ELISA had an OD below 0.8 (n=22). The OD cut-off value for positive detection by the sucrose gradient concentration method or the RidaQuick dipsticks has not previously been reported.

A problem with the comparison of fecal diagnostic methods is the lack of a true gold standard reference method (Srijan *et al*., 2005; Weitzel *et al*., 2006). Previous studies have used a combination of DFA, microscopy and repeated coproantigen test results as the reference standard. Only microscopy is considered to have 100% specificity, despite possible human error. Furthermore, coproantigen assays and microscopic analysis are testing for different items/end-point variables. The results from coporantigen tests should be evaluated against cases of known infection status (positive or negative) and not by the presence or absence of cysts such as with microscopy or DFA.

In this study, cyst quantification was performed using microscopic analysis and compared to ELISA absorbance values. There was no significant correlation between these values. This could be due to a lack of spread in the ELISA absorbance values; 93% of the 15 samples had an absorbance value of 2.9-3.0

indicating maximal or near-maximal absorbance. However, the results are consistent with the notion that ELISA can be considered a semi-quantitative assay. Only the samples with high absorbance readings were found to be positive by microscopic analysis. A limitation of using ELISA as a quantitative method is the maximal absorbance level of 3.0. This level is likely too low; a higher ceiling is necessary for the cases of giardiasis with a higher intensity of infection. Handoussa *et al* (2005) compared ELISA OD (Novum Diagnostics) readings to fecal cyst counts from people with confirmed giardiasis (n=83). No significant correlations were found. Vidal & Catapani (2005) reported ELISA intensity scores according to the degree of colour change (grading for positive results were from + to ++++ with the former having the highest colour and antigen intensity). The ELISA results were compared to microscopy (using sedimentation and the zinc-sulphate flotation method). A greater proportion of false-negative scores by microscopy occurred in the samples with the lowest positive intensity (+ and ++) by ELISA. This supports the idea that ELISA antigen detection is correlated to cyst count. Similarly, Johnston *et al* (2003) demonstrated that the ProSpecT ELISA had a greater number of false negative results when low cyst counts were reported by DFA. Addiss *et al* (1991) found a significant correlation between the OD from ProSpecT ELISA and cyst count (r^2=0.66, p=0.001). The correlation included false negative results by microscopy (n=20) resulting in a weaker correlation. More standardized testing to determine the correlation between ELISA OD and fecal cyst counts is needed. Further dilution and repeat ELISA analysis of the samples exceeding the 3.0 absorbance would be more costly and time consuming, but would facilitate the exploration of the concept of intensity-of-infection with the quantitative coproantigen approach.

The stool samples with a high ELISA absorbance value yet a negative detection by microscope could be explained by the inconsistency of fecal mass due to variation in stool quality. An important observation during microscopic analysis was that many stool samples contained large food particles that resulted in relatively less fecal matter in the slurry that was then concentrated and analyzed.

This may have been a factor resulting in the low sensitivity of the microscope method, which involved measuring out a mass of 0.5g of stool sample for analysis. This could also explain the lack of a significant correlation between cyst count and absorbance values. The ELISA and dipstick assays would be less affected by stool quality since the amount used in each assay is considerably less compared to the microscope method (0.2-50mg vs 500mg) because they detect coproantigens and not whole cysts.

4.5 Conclusion

Among the 3 methods evaluated, ELISA is the most sensitive, practical and feasible method, but it is also the most expensive at ~$9.50 CND (single well reading) per specimen, as compared to ~$5.50 CND for the dipstick method. ELISA has potential as a quantitative assay when the OD is measured with a reader but the maximal absorbance level needs to be higher and a standardized procedure for the comparative cyst counts is necessary. Dipsticks are a quick, easy to use method and could be useful in field settings for the detection of *Giardia*. Microscopic analysis is tedious and inconvenient. The sensitivity of microscopic analysis could be improved by adjusting the quantity of stool filtered and concentrated according to stool quality. Once the basic instrument (microscope) is in place, it has the lowest materials cost per specimen, ~$0.30 CND, if the hourly cost of highly trained labor is not included.

4.6 Tables

Table 4-1: Summary of *Giardia* Detection in Fecal Samples from ELISA, Dipstick and Microscopic Analysis

	Full series[a] (n=99)		Truncated series[b] (n=75)		
	ELISA	Dipstick	ELISA	Dipstick	Microscope
Number of Positive Samples	36	20	28	18	15
Number of Negative Samples	63	79	47	57	60
Total Number of Samples	99	99	75	75	75

[a]The full series includes 99 stool samples that were analyzed by ELISA and dipstick, 75 of those samples were also analyzed by microscope resulting in the [b]truncated series.

Table 4-2: Agreement Among Various Combinations of Fecal Tests for *Giardia* Detection

Test Combination	Sample Results in Agreement (%)
ELISA & Dipstick (n=99)[a]	79.8
ELISA & Dipstick (n=75)[b]	84.0
ELISA & Microscope (n=75)[b]	78.7
Microscope & Dipstick (n=75)[b]	89.3
All 3 Methods (n=75)[b]	74.7

[a]The full series includes 99 stool samples that were analyzed by ELISA and dipstick, 75 of those samples were also analyzed by microscope resulting in the [b]truncated series.

Table 4-3: ELISA versus Dipstick *Giardia* Diagnostic Results (n=99)

	ELISA Positive	ELISA Negative	Total
Dipstick Positive	18	2	20
Dipstick Negative	18	61	79
Total	36	63	99

Table 4-4: ELISA versus Microscope *Giardia* Diagnostic Results (n=75)

	ELISA Positive	ELISA Negative	Total
Microscopy Positive	15	0	15
Microscopy Negative	13	47	60
Total	28	47	75

Table 4-5: ELISA versus Dipstick *Giardia* Diagnostic Results (n=75)

	ELISA Positive	ELISA Negative	Total
Dipstick Positive	17	1	18
Dipstick Negative	11	46	57
Total	28	47	75

Table 4-6: Sensitivity, Specificity, PPV and NPV of Microscope and Dipstick Tests for *Giardia* Detection in Stool Samples using ELISA as Gold Standard

	Microscope (n=75)	Dipstick (n=75)	Dipstick (n=99)
Sensitivity	53.6	60.7	50.0
Specificity	100	97.9	96.8
PPV	100	94.4	90.0
NPV	78.3	80.7	77.2

Table 4-7: Comparison of ELISA Absorbance Values, Rida-Quick Dipstick Results and Microscopic Analysis for Diagnosis of Giardial Coproantigen or Cysts in Stool Samples

ELISA Absorbance Reading*	Dipstick	Microscope
3.0	+	+
3.0	+	N/A
3.0	+	+
3.0	+	-
3.0	+	+
3.0	+	+
3.0	+	-
3.0	+	+
3.0	+	+
3.0	-	+
3.0	+	-
3.0	+	+
3.0	+	+
3.0	+	+
3.0	+	+
2.9	+	N/A
2.9	+	+
2.9	+	+
2.9	+	+
2.8	-	N/A
2.6	-	-
2.5	-	N/A
2.5	+	-
2.2	-	-
1.7	-	+
0.77	-	N/A
0.51**	-	-

*maximum absorbance reading = 3.0;** all dipstick and microscope analysis below this absorbance reading were negative except for 2 false positive samples by the dipstick test; an ELISA absorbance value ≥0.05 is considered positive

Table 4-8: Comparison of ELISA Absorbance Values vs Microscopic Cyst Count of Stool Samples Positive for *Giardia* (n=15)

ELISA Absorbance Reading*	Cyst Count (cysts/g x10 000)
2.9	2.000
1.7	2.083
3.0	4.000
3.0	4.000
3.0	8.250
2.9	9.750
3.0	16.250
3.0	24.000
2.9	26.250
3.0	34.286
3.0	36.250
3.0	41.250
3.0	50.000
3.0	83.000
3.0	348.750

Spearman r=0.31, p=0.26 (2-tailed) non-significant

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CHAPTER 5: CONCLUSION

RECOMMENDATIONS FOR PARAMETERS FOR DESIGN OF A GANGLIOSIDE INTERVENTION STUDY TO SUPPRESS GIARDIAL INFECTION IN GUATEMALAN PRE-SCHOOL CHILDREN

5.1 Introduction

The objective of this chapter is to determine the most cost-effective sample size and methodology for a clinical trial evaluating gangliosides as a treatment agent for giardiasis. In addition to sample size estimations, this chapter will discuss recommendations for the number of stools to collect, choice of *Giardia* diagnostic method, ganglioside supplementation format and study staff requirements. The chapter includes a budget estimate of all the related field study costs. The study design recommendations reported in this chapter are based on a field research experience in Quetzaltenango, Guatemala conducted mainly in August and September, 2009 and 2010.

5.2 Sample Size Estimation

5.2.1 Statistical Analysis

A sample size estimation using Fischer's exact test for dichotomous data was conducted to establish the appropriate sample size needed to determine whether ganglioside treatment significantly reduces the proportion of the sample population with giardiasis from baseline to end-of-study (power=0.80, alpha=0.05). Different sample sizes were estimated based on increasing effect of ganglioside treatment (Table 5-3a). The number of required *Giardia*-positive subjects per group is reported. The corresponding total number of subjects per group (treatment arm: 1. ganglioside or 2. placebo) is reported for *Giardia* prevalence rates of 30%, 40% and 45%. The *Giardia* prevalence rates are based on the *Giardia* prevalence rates reported in Table 5-1 as well as previous unpublished results (Appendix C). In Table 5-1, there are different *Giardia* proportion rates reported depending on whether 1 or 2 stool samples were

collected. The *Giardia* proportion rates for collection of 2 stool samples are considered to be the true values, since collection of 1-stool sample results in under-detection of giardiasis. Therefore the 2-stool sample proportion rates for self-clearance and new infection of *Giardia* were used in the sample size estimation.

In the *Giardia* eradication design (Table 5-3a), the proportion of children self-clearing from *Giardia* (0.20) was used indicating that approximately 20% of the *Giardia*-positive subjects at baseline will become *Giardia*-negative without treatment by end-of-study. The added effect of ganglioside supplementation at eradicating giardiasis, symbolized as "Δ", was incorporated into the calculation with values ranging from a weak effect (Δ=0.20) to a strong effect (Δ=0.60). In the *Giardia* suppression design (Table 5-3b), the proportion of children newly infected with *Giardia* (0.20) was used indicating that approximately 20% of the *Giardia*-negative subjects at baseline will become *Giardia*-positive by end-of-study, if no intervention is in place. Sample size estimations for the *Giardia* suppression design were calculated for ganglioside supplementation periods lasting 4- (Table 5-3b), 8- (Table 5-3c) and 12- (Table 5-3d) weeks in duration. The *Giardia* new infection rate of 20% was assumed to occur at 4-week intervals affecting 0.20 of all non-infected subjects. Since the number of non-infected subjects would decrease as the original pool of subjects became infected over time, the number of new infections occurring in the second and third 4-week intervals (weeks 8 and 12, respectively) would decrease accordingly.

The drop-out rates reported in Table 5-2 remain below 30% up through collection of the fourth and final stool sample, of which less than half of the enrolled subjects complied. Appropriate timing of the clinical trial and other planning could help to reduce drop-out rates (discussed further under 'Daycare Center Screening'). Table 5-3e lists a summary of group sample sizes accounting for a 30% drop-out rate.

5.2.2 Results of Sample Size Estimation

Eradication of Giardiasis

If ganglioside supplementation for a period of 4-weeks had a large effect (Δ=0.80) on eradication of giardiasis, the minimum number of *Giardia*-positive subjects required per group would be 11. For *Giardia* prevalence rates of 30%, 40% and 45%, the corresponding total number of subjects (*Giardia*-positive and *Giardia*-negative) per treatment group would be 37, 28 and 24.

If ganglioside supplementation had a moderate effect (Δ=0.60) on eradication of giardiasis, the minimum number of *Giardia*-positive subjects required per group would be 20. For *Giardia* prevalence rates of 30%, 40% and 45%, the corresponding total number of subjects (*Giardia*-positive and *Giardia*-negative) per treatment group would be 67, 50 and 44.

If ganglioside supplementation had a small effect (Δ=0.40) on eradication of giardiasis, the minimum number of *Giardia*-positive subjects required per group would be 40. For *Giardia* prevalence rates of 30%, 40% and 45%, the corresponding total number of subjects (*Giardia*-positive and *Giardia*-negative) per treatment group would be 133, 100 and 89.

Suppression of New Infection of Giardiasis

If ganglioside supplementation for a period of 4-weeks had a large effect (Δ=0.80) on suppression of new infection of giardiasis, the minimum number of *Giardia*-negative subjects required per group would be 76. For *Giardia* prevalence rates of 30%, 40% and 45%, the corresponding total number of subjects (*Giardia*-negative and *Giardia*-positive) per treatment group would be 109, 127 and 138.

If ganglioside supplementation for a period of 4-weeks had a moderate effect (Δ=0.60) on suppression of new infection of giardiasis, the minimum number of *Giardia*-negative subjects required per group would be 146. For *Giardia* prevalence rates of 30%, 40% and 45%, the corresponding total number of subjects (*Giardia*-negative and *Giardia*-positive) per treatment group would be 209, 243 and 265.

If ganglioside supplementation for a period of 4-weeks had a small effect (Δ=0.40) on suppression of new infection of giardiasis, the minimum number of *Giardia*-negative subjects required per group would be 354. For *Giardia* prevalence rates of 30%, 40% and 45%, the corresponding total number of subjects (*Giardia*-negative and *Giardia*-positive) per treatment group would be 506, 590 and 644.

Extending the ganglioside supplementation period from 4-weeks to 8 and 12 weeks would help to decrease sample size substantially.

5.2.3 Discussion of Sample Size Estimation

Compared to the *Giardia* eradication study design, the suppression of new infection of giardiasis design requires a much larger sample size. A higher *Giardia* prevalence rate affects the sample size of each study design in an opposite manner; a high *Giardia* prevalence rate reduces the required sample size for the *Giardia* eradication design and increases the required sample size for the suppression of new infection design.

Increasing the duration of ganglioside supplementation for the suppression of new infection design could be a useful strategy to minimize sample size. If ganglioside supplementation has a moderate effect (Δ=0.60) on suppression of new infection, a 12-week study would only require approximately 69-87 subjects per group compared to 209-265 subjects needed per group for a 4-week supplementation period.

A conservative group sample size estimation for the eradication of *Giardia*, accounting for a potential 30% drop-out rate and assuming a *Giardia* prevalence rate between 30-40%, would be between 57-87 subjects per treatment group (Table 5-3e) for a total sample size of 114-174.

A conservative group sample size estimation for the suppression of new infection of giardiasis, accounting for a potential 30% drop-out rate and assuming a *Giardia* prevalence rate between 30-40%, would be between 272-345, 129-163 or 90-113

subjects per treatment group for ganglioside supplementation periods of 4-, 8- and 12-weeks duration (Table 5-3e). Total sample size would therefore be from 180 to 690 subjects.

5.3 Ideal Number of Stools to Collect

People with giardiasis have sporadic shedding of cysts resulting in under-detection of infection when using coproantigen or stool examination. A greater number of stools examined per subject increases the probability of *Giardia* detection. This finding is demonstrated in Table 5-1, when comparing the baseline proportion of children infected with *Giardia* of the 4-stool cohort (0.44) in which 2 stool samples were collected per week, in contrast to the much lower baseline proportion of children infected with *Giardia* of all subjects (0.34) when only 1 stool sample was collected per week. A higher detected rate of *Giardia* results in a lower sample size necessary for the clinical trial testing for eradication of giardiasis.

The collection of 2 stool samples per week gives a more complete and accurate picture of the prevalence situation. This information is important when comparing the proportion of children with giardiasis from baseline to end-of-study. For example, a subject with a false negative stool sample at baseline could mask a protective effect of ganglioside treatment. In contrast, with collection of 2 stool samples per week there is a smaller probability of a false negative stool sample.

The difficulty with collection of a higher number of stool samples per week is that more effort is needed from the daycare staff, the subjects and their parents. However, in the 2010 study one daycare demonstrated that it is feasible to have all children give 2 stool samples per week. In this daycare, 19 out of 20 children gave the requisite 4 stool samples; the 20th child gave 3 of the 4 stool samples. To maximize subject participation, stool samples should be collected during daycare hours. This could be done by utilizing the mothers who are already employed by the daycare to collect fecal samples during the day. The mothers

generally assist the younger children (~4yrs and under) to use the washroom, and are a familiar figure to the children attending daycare. Instead of being paid, the mothers could be given thank-you gift baskets.

The parents are another factor impacting full stool participation. During the information sessions with the daycares, it would be useful to gauge parent participation by taking a moment to discuss with the group of parents whether anyone anticipates difficulty obtaining 2 stool samples per week. Most parents are eager to participate, although problems have occurred in homes with other social problems. The participation rates of each daycare could be assessed if a screening week is incorporated into the study (discussed further in Section 5.5*)*. Participation is also affected by current events and weather. In the 2010 study, 79% of enrolled subjects were able to give 2 stool samples per week during the baseline week (Table 5-2). The decrease in participation during week 4 (only 43% of enrolled subjects submitted both stool samples) was mainly due to the national holiday (Independence Day) and heavy rains during September. Choose to conduct the study when there are 5 consecutive weeks of no national holidays or important religious events. The daycare school year begins in January and ends in late November. In Guatemala, the wet season (April to October) generally has more unpredictable weather which could impact daycare attendance. February and March would have more predictable, dryer weather but may also have lower prevalence rates of *Giardia*. Cifuentes *et al*. (2004) found higher *Giardia* prevalence rates during June through October (wet season) vs November through May (dry season) among children living in Mexico City.

Finally, the use of an interactive poster tracking stool sample collection for each child is an easy strategy to increase motivation among the participating children and to visually remind the daycare staff of the study. The poster can be colourful, using stickers to indicate submitted stool samples and be displayed at each daycare.

5.4 Choice of Diagnostic Method

5.4.1 List of Selected Methods

The 3 methods of Giardia detection in feacal samples include 1) microscopic analysis of *Giardia* cysts using the sucrose gradient concentration method 2) the RidaQuick *Giardia* dipstick test and 3) the ProSpecT-*Giardia*-EZ-Microplate ELISA.

5.4.2 Summary of the Sensitivity and Specificity

As discussed in Chapter 3, the ELISA was used as the standard of comparison for evaluation of sensitivity and specificity of the other 2 methods. Compared to ELISA, the microscope had a sensitivity of 53.6% and specificity of 100%. The dipsticks had a sensitivity ranging between 50.0-60.7% and specificity ranging between 96.8-97.9%, depending on the number of stool samples used from comparison (n=75 or n=99).

5.4.3 Summary of the Cost and Time Commitment

Excluding the cost of purchasing a microscope, the microscopic analysis has the lowest cost per sample ($0.30) and has the lowest total cost ($477 for 90 samples) despite the large time commitment for analysis (9 days compared to <1 day for dipstick or ELISA). Intensity of infection is an important factor. Changes in fecal cyst concentration after ganglioside supplementation should be calculated. This can be done by using ELISA particularly if standardized dilutions are done to provide a larger spread in OD readings. If suggested improvements are made to sample preparation for microscopy it is possible that cyst counts will be more reliable.

The dipstick test has a comparable total cost ($545) to microscopic analysis and offers considerably more flexibility; stools can be frozen, analysis (n=90) takes less than a day and can be done by any staff with basic laboratory training (very simple procedure).

The ELISA has the highest cost and analysis is limited to trained staff at *La Democracia* due to the use of the spectrophotometer. However, similar to the dipstick test, analysis of 90 samples can be done in less than a day and the stool samples can be fresh or frozen. *Giardia* antigen is detected by ELISA for up to four days following initiation of pharmaceutical treatment (Green *et al*., 1985). In comparison, cyst detection generally occurs for a shorter period of time (2 days) after initiation of treatment. This should not be a factor in the clinical trial since the ganglioside supplementation period is at least 21 days.

5.4.4 Recommendations

The total cost of the ELISA is almost double the amount per sample compared to the cost of the dipstick test or microscopic analysis. However, the detection sensitivity of ELISA is significantly higher. The sample size estimation calculations are dependent on the sensitivity of ELISA. Based on the information found from the current study, the recommendation for the future clinical trial is to use ELISA for all stool analysis. Microscopy should not be used. Dipsticks may serve a special screening purpose for selection of daycare centers (details below).

5.5 Daycare Center Screening

Screening daycare centers for giardiasis has 3 potential roles in the ganglioside clinical trial. The first use is to screen different daycare centers to select the daycares with the highest *Giardia* prevalence rates to participate in the study. Through this screening step, stool collection participation rates per daycare can be gauged. The third purpose of the screening is to stratify subjects according to infection status (negative or positive) prior to randomizing them to treatment or placebo. The results obtained for the first purpose (daycare screening) could be used for the third purpose (subject stratification). There are 3 potential scenarios for the screening phase (Figure 5-1). The additional cost, advantages and disadvantages are shown in Table 5-5.

5.5.1 Selection of Daycare Centers with High Giardia *Prevalence Rates*

Giardia prevalence rates are variable among different daycare centers (Appendix C). Half of the screened daycare centers may have *Giardia* prevalence rates equal or greater than 35% suggesting that 2 daycares will need to be screened for every daycare needed to participate. Due to reliance on single stool sample collection, it is likely that *Giardia* prevalence rates are actually higher than previously reported (see Appendix C for extrapolated data). A single stool sample could be used for screening if it is accepted that a single stool sample collection will consistently under-detect giardiasis.

5.5.2 Assessment of Stool Collection Participation Rates

In scenario 1, the single stool sample collection will give some indication of the participation rate of enrolled subjects from each daycare. Scenario 2 will indicate the percentage of children who provide 2 stool samples, a rate which can be highly variable among daycare centers. Scenario 3 will also give indication as to the participation rates of each daycare. A disadvantage with this set-up is the lack of time (approximately 2 days) to consider the different combinations of subject participation rate, *Giardia* prevalence rates and cookie delivery logistics for each daycare center.

5.5.3 Equal Distribution of Giardia-*Positive Subjects per Group*

There is a large range in the potential distribution of *Giardia*-positive subjects through randomization. The use of dipsticks to stratify *Giardia*-positive subjects results in modest improvements in the 95% confidence interval (Table 5-6). Unequal sample sizes could compromise the statistical significance of the study results. An alternative solution would be to conduct the ELISA test on all baseline stool samples immediately after collection to stratify and then randomize subjects according to positive or negative stool results. This method would ensure equal distribution *Giardia* carriers in each treatment group and would not result in any additional study costs (assuming that dipsticks are used in the screening phase).

5.5.4 Recommendations

Scenario 2 results in the consistent practice of collecting 2 stool samples per week and would help select the daycare centers most likely to give full stool collection participation. The timeline enables flexibility in that all the screened daycare centers can be fully evaluated before being committed to the study. Based on the wide confidence interval associated with dipstick-stratified randomization, it is recommended that the baseline stool samples of the participating daycare centers be analyzed immediately by ELISA and then stratified and randomized into groups.

5.6 Ganglioside Supplementation

5.6.1 Dosage

An exclusively breastfed infant at the age of 6 months may consume approximately 700 mL of breast milk/day (Islam *et al*., 2008). Mature human breast milk contains between 1.78-8.02 mg/L of lipid-bound sialic acid (LBSA) (Rueda, 2007). This suggests that a 6-month old exclusively breast-fed infant ingests between 2-11 mg of gangliosides per day from the mother's milk.
In the ganglioside clinical trial, children (aged 2-7 yrs) will be supplemented with a ganglioside-enriched milk fraction from bovine buttermilk powder that will be incorporated into an icing mixture, and sandwiched between 2 vanilla cookies. The concentration of ganglioside (mainly GM3 and GD3) in this buttermilk is approximately 0.02%. A daily dose of 5g of buttermilk will result in an intake of approximately 10mg/d of ganglioside (mainly GM3 and GD3). The amount of intestinal fluid present in children is estimated to be between 70-100mL/kg body weight. The mean body weight of all participating children in 2010 (n=46) was 14.5±2.7 kg. The estimated mean volume of intestinal fluid is between 1015-1450mL. Table 5-7 indicates the estimated range of daily intestinal ganglioside concentration (μg/mL) according to 3 categories of body weight and assuming a daily ganglioside dose of 10mg. In 2 of the 3 body weight categories the desired intestinal ganglioside concentration is achieved if the conservative intestinal fluid

volume coefficient is used (70mL/kg body weight). In contrast, none of the body weight categories achieve the desired intestinal ganglioside concentration if the higher intestinal fluid volume coefficient is used (100mL/kg body weight).
In pharmaceutical treatments, dosing is designed to maintain a constant concentration of the active ingredient. To facilitate this, the treatment is usually consumed or applied at repeated intervals of time. It is possible that ganglioside treatment would be optimized if given more than once per day. In the daycare context, a feasible compromise would be to administer the cookies twice a day (once in the morning and once in the afternoon). Ganglioside dosages could be adjusted accordingly. Ganglioside cookies could also be given at night by the parents to minimize any potential decrease in effectiveness associated with food intake that would occur during daytime hours.

5.6.2 Placebo

A small amount of placebo (5g/day) will be mixed into the icing of the cookies intended for the placebo group. Skim milk powder was chosen as the placebo because it has the lowest ganglioside concentration compared to whole milk or buttermilk powder. To minimize a potential confounding effect associated with increased micronutrient intake, a non-fortified skim milk powder should be used. A disadvantage of skim milk powder is that its appearance is more granulated and more white compared to the ganglioside-enriched buttermilk fraction. However, once incorporated into the icing mixture, the appearance and taste of the cookies are indistinguishable.

5.6.3 Storage

The ganglioside-enriched buttermilk fraction should be stored in a vacuum sealed bag, placed inside an opaque bag and kept in the freezer (-20°C) to minimize deterioration of ganglioside. Stored ganglioside-enriched buttermilk had no detectible loss in concentrations when stored under similar conditions for a period of 1-month. The ganglioside concentration of the enriched buttermilk was initially 624 mg/kg and after 1 month was 620 mg/kg. In this study, ganglioside

was placed in air-tight ziplock instead of a vacuum sealed bag suggesting that stability of ganglioside can be maintained under less stringent conditions. Once the ganglioside containing package was opened (and then closed and kept in the freezer at -20°C), minimal loss was observed after a period of 1-week (the ganglioside concentration was 584 mg/kg compared to the original value of 620 mg/kg). The decrease in ganglioside concentration was within the error range and not statistically significant. Storage at warmer temperatures for a period of 1 week also had minimal effect on the concentration of ganglioside (the concentration after 1 week when stored at 5°C was 549 mg/kg; 21°C was 515 mg/kg; and 37°C was 534 mg/kg).

A conservative approach to ensure minimal loss of ganglioside would be to package the ganglioside-enriched buttermilk fraction based on the weekly amount (g) needed and to store in the freezer.

5.6.4 Cookies

The storage effects on the taste and texture of premade cookies was evaluated. Cookies were made, individually packaged in aluminum foil and placed together (n=3) inside a sealed ziplock bag and stored a) at room temperature, b) in the refrigerator or c) in the freezer for 2 weeks. After 2 weeks, a fresh batch of cookies was made to enable comparison. The cookies stored in the freezer maintained the crunchy texture of the cookie and the soft and smooth texture of the icing. In contrast, storage at room temperature or in the refrigerator resulted in a slightly soggier cookie and dryer icing due to moisture absorbance from the icing. The taste of the cookies was not affected by storage condition, and was determined as good in all 3 scenarios.

The impact of the duration of storage of premade cookies on the concentration of ganglioside has not been evaluated. Therefore, it is recommended that premade cookies be made no more than 2 days in advance of the day in which they will be consumed.

Cookies may be a problematic food vector for the ganglioside-enriched buttermilk fraction. Cookies would be useful for take-home supplementation since they do not require refrigeration but if a higher dose of ganglioside is needed, the subjects could get tired of eating 2 large cookies per day for a period of 28 days. An alternative to cookies is to add and mix a standardized scoop of the ganglioside-enriched buttermilk fraction to the subject's daily meals, snacks or drinks prior to consumption. The children attend daycare from 8am to 4pm. During this time they consume 2 meals and 2 snacks. The meals and snacks frequently include warm cereals (oatmeal or a corn based cereal), milk or cereal-based beverages, soups, sauces and beans. Any liquid based, creamy food or drink would serve as an appropriate food vector for the 1/2 tbsp scoop of ganglioside-enriched buttermilk fraction.

5.7 Study Staff Requirements

The necessary research staff for the clinical trial includes a graduate student or CeSSIAM staff responsible for food laboratory activities, a graduate student or CeSSIAM staff responsible for stool collection and analysis, and a local project coordinator. The non-research staff required includes field assistants for cookie production, teachers and moms working at the daycare centers and a physician to oversee ganglioside supplementation and metronidazole treatment.

5.8 Study Costs (Table 5-9)

5.8.1 Fixed Costs

The fixed costs for the clinical trial are independent of the sample size and the number of stool samples collected per week. The fixed costs include the IRB fee, the cost of renting food lab space, the cost of the freezer, and the cost of the main study staff from CeSSIAM and the University of Alberta (graduate students, project coordinator).

5.8.2 Grouped Costs (costs that change with varying increments in response to sample size)

The grouped costs are the costs that will increase at irregular intervals due to an increase in sample size or in the number of stools collected. Group costs include certain staff (the study physician, the daycare teachers and moms, the food lab assistants), stool analysis kits (ELISA and dipsticks) and metronidazole treatment for the screened daycare centers. An increase in sample size will increase the workload of the study physician since there will be a higher number of children to treat with metronidazole. However, the increase in the physician's workload may only become apparent if the sample size is increased substantially such as after every 50 additional subjects. Similarly, a higher sample size means more participating daycares. For every increase in approximately 15-20 children, the number of participating daycares will increase by 1 resulting in approximately 3 additional daycare staff (1 teacher and 2 moms). The teachers and moms will not be paid directly, however a gift valuing approximately $75 CND per person will be purchased. A higher sample size means more cookies will need to be produced. An increase in 20-25 children will result in an additional food lab student.

The increase in stool samples (due to either the use of 2 stool samples per week or an increase in sample size) will increase the number of stool analysis kits required. Kits are sold containing a set number of samples and partial use of a kit will not result in a decrease in cost. The ELISA kit holds enough wells to analyze approximately 90 samples and the dipstick kit can analyze 25 samples. If 4 stool samples are needed per subject, then an additional ELISA kit will need to be purchased for the 23^{rd}, 46^{th}, 68^{th}, 91^{st} and 113^{th} subject. If 2 stool samples are needed per subject, then an additional ELISA kit will need to be purchased for the 46^{th} and 91^{st} subject. The cost of ELISA analysis is based on the number of kits used, partial or complete. Dipsticks are available in smaller packages and are less costly and so the amount of excess money spent on unused dipstick strips is much smaller. Since ELISA is the highest non-fixed cost, basing sample size on maximizing complete usage of the ELISA kits could help minimize costs.

Children that were diagnosed as positive for *Giardia* from the daycare screening phase will need to be treated with metronidazole. Treatment costs for all the screened daycare centers would not be very high. Each subject needing treatment would need 2x250 mL bottles or a cost of $1.63. The prevalence rate for the screened daycare centers using dipsticks would be between 5-15%. If 3 daycares were screened with a combined n=60 and a mean 10% prevalence rate, there would be an additional 6 children needing metronidazole treatment (cost of approximately $10).

5.8.3 Per Child Costs

The costs that will increase directly in proportion to the number of subjects participating include items such as cookies, stool collection kits and metronidazole. Cookie costs assumed a mean supplementation period of 28 or 29 days (1 or 2 stool samples per week) requiring 1-cookie per day. The cost of 1 cookie is approximately $0.22. The ingredient costs are summarized in Table 5-8. The cost of metronidazole is based on the assumption that ganglioside treatment will be 60% effective at eradicating and suppressing giardiasis. Since only half of all subjects will be in the ganglioside treatment group, approximately 32% of the initial sample size will have giardiasis at end-of-study, assuming baseline prevalence rate of 40%. Therefore the per child cost for metronidazole treatment could be 32% x $1.63, equal to $0.52 per child.

5.9 Conclusion

This concluding chapter discussed study design, choice of stool analysis methods, number of stools to collect, staff roles and responsibilities, and field study costs. The choice of stool analysis method strongly favours ELISA due to its superior sensitivity. The number of stools to collect should be 2 and an extra screening week should be included with the collection of 2 stool samples analyzed by dipsticks. A sample size of approximately 65 subjects per group would have sufficient power to detect a protective effect of ganglioside at eradicating

giardiasis in a sample population with a 40% prevalence rate of giardiasis. The total cost of the above described study would be between $32 637 and $40 637 CND (approximately $17 287 being grouped and per child costs, and $15 350-23 350 being fixed costs). It is recommended that a pilot study be conducted at a daycare center with very high prevalence of *Giardia* to determine the appropriate ganglioside dose. It is possible that supplementation twice a day (ie 9am and 3pm) would be more effective than a single dose.

5.10 Tables & Figures

Table 5-1: Baseline Proportion Rates of *Giardia* Infection in Children (2-7 yrs) Attending Daycare in Guatemala

	Subjects with 2 stool samples collected per week (n=23)	Subjects with 1 stool sample collected per week (n=38)[a]
Baseline total proportion of children infected with *Giardia* (n)	0.44 (10)	0.34 (13)
Baseline total proportion of children not infected with *Giardia* (n)	0.56 (13)	0.66 (25)
Self-clearance and new infection events		
Proportion of infected children at baseline who self-cleared *Giardia* by end-of-study (n)	0.20 (2/10)	0.31 (4/13)
Proportion of children newly infected with *Giardia* by end-of-study (n)	0.15 (2/13)	0.24 (6/25)

[a]For calculation purposes, only the first stool sample per week was considered for subjects who gave 2 stool samples during a single week.

Table 5-2: Stool Sample Collection Rates from Daycare Centers of Enrolled (n=53) and Participating (n=48) Subjects During Baseline and End-of-Study Collection Weeks

	Baseline (week 0)		End-of-study (week 4)	
	Sample 1/2	Sample 2/2	Sample 1/2	Sample 2/2
Number of Subjects	48	42	38	23
% of Participating Subjects (n=48)	100%	88%	79%	48%
% of Enrolled Subjects (n=53)	91%	79%	72%	43%

Table 5-3a: Sample Size Estimations for a Clinical Study Evaluating the Effect of 4-Week Ganglioside Supplementation on Eradication of Giardiasis using Fischer's Exact Test

Eradication of Giardiasis					
Baseline proportion of children infected with Giardia *per group*		N			
Proportion of infected children at baseline who self-cleared Giardia *by end-of-study*		N*(0.20)			
Ganglioside Treatment Effect		Δ			
End-of-Study proportion of children infected or not infected with Giardia *per group*		Infected	Not-Infected		
	Placebo	N*(0.80)	N*(0.20)		
	Treatment	N*(0.80-Δ0.80)	N*(0.20+Δ0.80)		

	End-of-Study proportion of children infected with Giardia *per group*		*Sample Size Estimation* [a]			
Δ	Proportion of Infected Subjects in the Treatment Group:	Proportion of Infected Subjects in the Placebo Group:	Number of *Giardia*-Positive Subjects Needed per Group:	*Group Sample Size Assuming the following* Giardia *Prevalence Rates*		
				30%	40%	45%
0.40	N*(0.48)	N*(0.80)	40	133	100	89
0.50	N*(0.40)	N*(0.80)	27	90	68	60
0.60	N*(0.32)	N*(0.80)	20	67	50	44
0.70	N*(0.24)	N*(0.80)	14	47	35	31
0.80	N*(0.16)	N*(0.80)	11	37	28	24

N=Group sample size; Δ=ganglioside treatment effect; [a] Sample size estimation was completed using Fischer's Exact Test for dichotomous data to detect a difference between 2 proportions.

Table 5-3b: Sample Size Estimations for a Clinical Study Evaluating the Effect of 4-Week Ganglioside Supplementation on Suppression of Giardiasis using Fischer's Exact Test

Suppression of New Giardia *Infection using a 4-week supplementation period*			
Baseline proportion of children not infected with Giardia *per group*		N	
Proportion of children newly infected with Giardia *by end-of-study*		N*(0.20)	
Ganglioside Treatment Effect		Δ	
		Not Infected	Infected
End-of-Study proportion of children infected or not infected with Giardia *per group*	Placebo	N*(0.80)	N*(0.20)
	Treatment	N*(0.80 + 0.20Δ)	N*(0.20 - 0.20Δ)

	End-of-Study proportion of children not infected with Giardia *per group*		Sample Size Estimation [a]			
Δ:	Proportion of Not Infected Subjects in the Treatment Group:	Proportion of Not Infected Subjects in the Placebo Group:	Number of *Giardia*-Negative Subjects Needed per Group:	Group Sample Size Assuming the following *Giardia* Prevalence Rates:		
				30%	40%	45%
0.40	N*(0.88)	N*(0.80)	354	506	590	644
0.50	N*(0.90)	N*(0.80)	219	313	365	398
0.60	N*(0.92)	N*(0.80)	146	209	243	265
0.70	N*(0.94)	N*(0.80)	103	147	172	187
0.80	N*(0.96)	N*(0.80)	76	109	127	138

N=Group sample size; Δ=ganglioside treatment effect; [a] Sample size estimation was completed using Fischer's Exact Test for dichotomous data to detect a difference between 2 proportions.

Table 5-3c: Sample Size Estimations for a Clinical Study Evaluating the Effect of 8-Week Ganglioside Supplementation on Suppression of Giardiasis using Fischer's Exact Test

Suppression of New Giardia *Infection using an 8-week supplementation period*	
Baseline proportion of children not infected with Giardia *per group*	N
Proportion of children newly infected with Giardia *by end-of-study*	=N*[0.20 + (0.80*0.20)] =N*(0.20 + 0.16) =N*(0.36)
Ganglioside Treatment Effect	Δ

		Not Infected	Infected
End-of-Study proportion of children infected or not infected with Giardia *per group*	Placebo	N*(0.64)	N*(0.36)
	Treatment	N*(0.64+ 0.36Δ)	N*(0.36-0.36Δ)

	End-of-Study proportion of children not infected with Giardia *per group*		Sample Size Estimation [a]			
Δ:	Proportion of Not Infected Subjects in the Treatment Group:	Proportion of Not Infected Subjects in the Placebo Group:	Number of *Giardia*-Negative Subjects per Group:	Group Sample Size Assuming the following *Giardia* Prevalence Rates		
				30%	40%	45%
0.40	N*(0.78)	N*(0.64)	178	254	297	324
0.50	N*(0.82)	N*(0.64)	105	150	175	191
0.60	N*(0.86)	N*(0.64)	69	99	115	125
0.70	N*(0.90)	N*(0.64)	47	67	78	85
0.80	N*(0.94)	N*(0.64)	34	49	57	62

N=Group sample size; Δ=ganglioside treatment effect; [a] Sample size estimation was completed using Fischer's Exact Test for dichotomous data to detect a difference between 2 proportions.

Table 5-3d: Sample Size Estimations for a Clinical Study Evaluating the Effect of 12-Week Ganglioside Supplementation on Suppression of Giardiasis using Fischer's Exact Test

Suppression of New Giardia *Infection using a 12-week supplementation period*						
Baseline proportion of children not infected with Giardia *per group*			N			
Proportion of children newly infected with Giardia *by end-of-study*			=N*[0.20 + 0.16 + (0.64*0.20)] =N*(0.20 +0.16 + 0.13) =N*(0.49)			
Ganglioside Treatment Effect			Δ			
End-of-Study proportion of children infected or not infected with Giardia *per group*			Not Infected	Infected		
	Placebo		N*(0.51)	N*(0.49)		
	Treatment		N*(0.51 + 0.49Δ)	N*(0.49 - 0.49Δ)		
	End-of-Study proportion of children not infected with Giardia *per group*		*Sample Size Estimation* [a]			
Δ:	Proportion of Not Infected Subjects in the Treatment Group:	Proportion of Not Infected Subjects in the Placebo Group:	Number of *Giardia*-Negative Subjects per Group:	Group Sample Size Assuming the following *Giardia* Prevalence Rates:		
				30%	40%	45%
0.40	N*(0.71)	N*(0.51)	102	146	170	185
0.50	N*(0.76)	N*(0.51)	65	93	108	118
0.60	N*(0.80)	N*(0.51)	48	69	80	87
0.70	N*(0.85)	N*(0.51)	34	49	57	62
0.80	N*(0.90)	N*(0.51)	25	36	42	45

N=Group sample size; Δ=ganglioside treatment effect; [a] Sample size estimation was completed using Fischer's Exact Test for dichotomous data to detect a difference between 2 proportions.

Table 5-3e: Summary of Sample Size Estimations for Eradication and Suppression of Giardiasis using Fischer's Exact Test and Assuming a Moderate[a] Effect from Ganglioside Supplementation

Study Design	Group Sample Size			Group Sample Size after Incorporating a Subject Drop-Out Rate of 30%		
	Giardia *Prevalence Rate*			Giardia *Prevalence Rate*		
	30%	40%	45%	30%	40%	45%
Giardia *Eradication*						
4-week supplementation	67	50	44	87	65	57
Suppression of New Infection of Giardia						
4-week supplementation	209	243	265	272	316	345
8-week supplementation	99	115	125	129	150	163
12-week supplementation	69	80	87	90	104	113

[a] A moderate effect of ganglioside supplementation was chosen as $\Delta = 0.60$.

Table 5-4: Summary of the Cost and Time Commitment of *Giardia* Diagnostic Methods using Stool Samples

Diagnostic Method	Cost per Sample (Kits and Supplies)	Cost of Analysis (Staff/Labour) for n=90 samples	Total Cost for n=90 samples
Microscopic Analysis	$0.30[a]	9 days at $50/day[b], assuming 10 fresh stool samples are available per day	$477
Dipstick	$5.50	5-6 hrs unskilled staff, ~$50/day	$545
ELISA	$9.50	$325 per kit[c] for spectrophotometer readings	$1180

[a]Excluding cost of microscope; [b]Based on a monthly salary of $1000 and 20 working days/month; [c]Assuming the exchange rate is 1 Guatemalan Quetzal = $0.13 Canadian Dollars and analysis is done at *La Democracia* laboratory in Quetzaltenango.

Table 5-5: Summary of the Costs Associated to *Giardia* Screening Scenarios for Selection of Participating Daycare Centers with the Highest *Giardia* Prevalence Rates

Scenario	Additional Cost[a]	Advantages/Disadvantages
1. One extra stool sample per child analyzed by dipstick	2N*$5.50	Most inexpensive option Provides moderate insight into participation rates An additional stool sample is required per subject (total of 5 per subject)
2. Two extra stool samples per child analyzed by dipstick	2N*$11.00	Provides good insight into participation rates Two additional stool samples are required per subject (total of 6 per subject)
3. No extra stool samples, all stool samples from screened daycares are analyzed by ELISA	2N*$19.00	Most expensive option Study supplementation begins immediately (within 2 days after screening) making the baseline week very hectic No additional stool samples are required (total of 4 per subject)

[a]Additional costs include any cost from dipstick or ELISA testing that is in addition to the ELISA testing during baseline and end-of-study for participating subjects; N=study sample size; 2N=the number of children needed to be screened in order to select appropriate participating daycare centers.

Table 5-6: Comparison of the Distribution of *Giardia*-Positive Subjects per Intervention Group using Randomization or Stratified Randomization

Group Sample Size	Ideal Number of *Giardia*-Positive Cases per Group (Using Prevalence of 40%)	Range of the Number of *Giardia*-Positive Cases per Group Using Randomization	Range of the Number of *Giardia*-Positive Cases per Group Using Stratified Randomization Based on Dipstick Results[a]
130	52	42-64 (32-49%)[b]	44-61 (34-47%)[b]

[a]Assuming dipsticks can detect 50% of cases; [b]Range in the *Giardia* prevalence rate per group based on the 95% confidence interval of the number of *Giardia* positive cases; source http://statpages.org/confint.html#Binomial.

Table 5-7: Body Weight, Intestinal Fluid and Ganglioside Dosage Calculations for Study Subjects

Body Weight Categories (kg)	Estimated[a] Daily Volume of Intestinal Fluid (mL)	Daily Ganglioside Dose Administered (mg)	Daily Intestinal Ganglioside Concentration (μg/mL)
8.0 - 12.0	560 - 1200	10	8.3 - 17.9
12.1 - 16.0	847 - 1600	10	6.3 - 11.8
16.1 - 20.0	1127 - 2000	10	5.0 - 8.9

[a]Estimation based on personal communication from a gastroenterologist, values extrapolated from adult intestinal fluid volumes of 100mL/kg body weight.

Table 5-8: Cookie Recipe Cost

Icing			
Ingredients	Weight	Cost (GQT)	Cost (CND)
1/2 cup of butter	220 g or (1/2 pound)	Q20.00	$2.60
3 cups of icing sugar	375 g	Q13.00	$1.69
2 tsp of vanilla	10 mL	Q 0.50	$0.07
2 tbsp milk	30 mL	Q 0.50	$0.07
Total	=600g or 30 servings	Q34.00	$4.42
Cost per Serving	1 tbsp (18g)	Q 1.20	$0.15
Cookie			
Maria Cookies	Box of 30	Q8.00	$1.04
	2 Cookies	Q0.50	$0.07
Total cost per cookie (2 Maria cookies + 1 tbsp icing)		*Q1.7 or $0.22*	

Table 5-9: Field Cost Summary of a 4-Week Ganglioside Supplementation Trial

Staff/Supplies/Fees/Gifts	Price (CND)	The Number of Subjects Corresponding to an Additional Increase in the Required Staff/Supply/Fees/Gifts[a]
Fixed Costs		
IRB fee	$1000	N/A
Cookie Graduate Student (3-5 months)	$4800-$8000	N/A
Stool Graduate Student (3-5 months)	$4800-$8000	N/A
Project Coordinator (Part-Time, 3-5 months)	$2400-$4000	N/A
Food Lab Space (3-5 months)	$2000	N/A
Freezer	$350	N/A
Sub-Total	*$15 350 - $23 350*	
Grouped Costs		
Physician	$500	50
1 Teacher & 2 Moms (per daycare)	$225	20
Undergraduate Cookie Staff	$500	25
ELISA	$900	22
ELISA Analysis	$665	22
Dipsticks	$137	12
Per Child Costs		
Cookies	$6.50	1
Stool Kits	$1.00	1
Metronidazole	$0.52	1

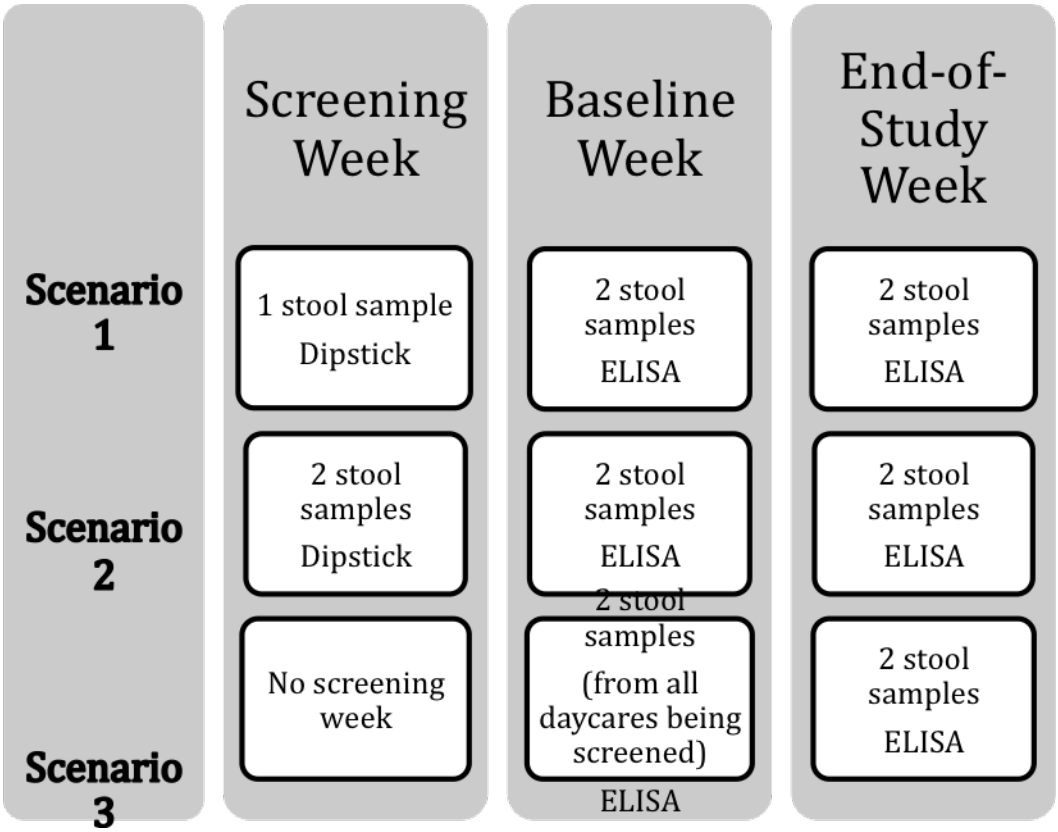
[a]Fixed Costs' are not affected by increases in sample size, the 'Grouped Costs' change in varying degrees in response to increases in sample size, the 'Per Child Costs' are already calculated to increase for every additional subject.

Table 5-10: Summary of Study Costs (Grouped Costs and Per Child Costs) According to Sample Size and a Moderate Effect[a] of Ganglioside Supplementation

Study Design	Total Sample Size			Study Cost According to Group Sample Size
	Giardia *Prevalence Rate*			40% Giardia *Prevalence Rate*
	30%	40%	45%	Grouped Costs + Per Child Costs
Giardia *Eradication*				
4-week supplementation	174	130	114	$17 287 CND
Suppression of New Infection of Giardia				
4-week supplementation	544	632	690	$83 677 CND
8-week supplementation	258	300	326	$40 110 CND
12-week supplementation	180	208	226	$28 030 CND

[a]A moderate effect of ganglioside supplementation on eradication/suppression of giardiasis was chosen as $\Delta = 0.60$.

Figure 5-1: Outline of Stool Collection Weeks and Choice of *Giardia* Diagnostic Method for Stool Analysis for Different Daycare Center Screening Scenarios



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APPENDICES

6-1a Appendix A: Demographic and Anthropometric Description (WHO 2006 Child Growth Standards) of Sample of Subjects Participating (n=48) in the Stool Collection

Mean Age (months)	54.1 ± 15.5
Females	N=27
Males	N=21

Anthropometrics	WAZ*	HAZ*	WHZ*
	WHO 2006	*WHO 2006*	*WHO 2006*
Ages			
<35.9 months (n=5)	-1.45±0.71	-2.54±0.74	-0.05±1.49
Female (n=3)	-1.10±0.33	-2.70±0.85	0.62±0.80
Male (n=2)	-1.98±0.93	-2.30±0.71	-1.06±2.05
36-47.9 months (n=13)	-1.08±1.29	-2.16±0.99	0.28±1.14
Female (n=8)	-1.05±1.25	-2.05±0.85	0.24±1.19
Male (n=5)	-1.14±1.50	-2.34±1.27	0.33±1.19
48-59.9 months (n=13)	-1.73±0.96	-2.65±0.70	-0.18±1.25
Female (n=7)	-1.75±0.55	-2.70±0.70	-0.10±0.81
Male (n=6)	-1.72±1.36	-2.60±0.76	-0.26±1.71
≥ 60 months (n=15)	-1.35±0.85	-1.73±1.03	-0.32±0.87**
Female (n=8)	-1.52±0.65	-1.80±0.95	-0.51±0.72
Male (n=7)	-1.16±1.05	-1.64±1.18	-0.09±1.04
	% Global Malnutrition (WAZ <-2 S.D.)*	*% Stunting (HAZ* <-2 S.D.)*	*% Wasting (WHZ* <-2 S.D.)*
	WHO 2006	*WHO 2006*	*WHO 2006*
Ages			
<35.9 months (n=5)	*20.0*	*60.0*	*20.0*
Female (n=3)	*0.0*	*66.7*	*0.0*
Male (n=2)	*50.0*	*50.0*	*50.0*
36-47.9 months (n=13)	*23.1*	*46.2*	*0.0*
Female (n=8)	*25.0*	*37.5*	*0.0*
Male (n=5)	*20.0*	*60.0*	*0.0*
48-59.9 months (n=13)	*23.1*	*84.6*	*7.7*
Female (n=7)	*28.6*	*85.7*	*0.0*
Male (n=6)	*16.7*	*83.3*	*16.7*
≥ 60 months (n=15)	*26.7*	*33.3*	*0.0*
Female (n=8)	*25.0*	*50.0*	*0.0*
Male (n=7)	*28.6*	*14.3*	*0.0*

*mean z-score ± S.D.;** mean z-scores of body mass index (BMI) for age; WHO=World Health Organization; WAZ=weight-for-age-z-score; HAZ-height-for-age-z-score; WHZ=weight-for-height-z-score; S.D.=standard deviation

6-1b Appendix A: Demographic and Anthropometric Description (CDC 2000 Child Growth Standards) of Sample of Subjects Participating (n=48) in the Stool Collection

Mean Age (months)	54.1 ± 15.5
Females	N=27
Males	N=21

Anthropometrics	WAZ*	HAZ*	WHZ*
	CDC 2000	*CDC 2000*	*CDC 2000*
Ages			
<35.9 months (n=5)	*-1.84*±0.93	*-2.25*±0.68	*-0.53*±1.50
Female (n=3)	-1.42±0.39	-2.38±0.83	0.14±0.82
Male (n=2)	-2.48±1.34	-2.06±0.58	-1.53±2.06
36-47.9 months (n=13)	-1.23±1.58	-1.83±1.05	-0.11±1.33
Female (n=8)	-1.17±1.55	-1.66±0.88	-0.13±1.42
Male (n=5)	-1.33±1.81	-2.10±1.33	-0.07±1.33
48-59.9 months (n=13)	-2.01±1.34	-2.36±0.74	-0.54±1.43
Female (n=7)	-1.91±0.69	-2.38±0.79	-0.42±0.91
Male (n=6)	-2.13±1.93	-2.34±0.76	-0.68±1.98
≥ 60 months (n=15)	-1.40±0.93	-1.59±1.00	-0.42±0.95
Female (n=8)	-1.63±0.88	-1.80±1.08	-0.55±0.78
Male (n=7)	-1.13±0.97	-1.34±0.91	-0.27±1.17

	% Global Malnutrition (WAZ <-2 S.D.)	*% Stunting (HAZ* <-2 S.D.)	*% Wasting (WHZ* <-2 S.D.)
	CDC 2000	*CDC 2000*	*CDC 2000*
Ages			
<35.9 months (n=5)	*20.0*	*60.0*	*20.0*
Female (n=3)	*0.0*	*66.7*	*0.0*
Male (n=2)	*50.0*	*50.0*	*50.0*
36-47.9 months (n=13)	*30.8*	*38.5*	*7.7*
Female (n=8)	*25.0*	*25.0*	*12.5*
Male (n=5)	*40.0*	*60.0*	*0.0*
48-59.9 months (n=13)	*46.2*	*29.2*	*7.7*
Female (n=7)	*57.1*	*57.1*	*0.0*
Male (n=6)	*33.3*	*83.3*	*16.7*
≥ 60 months (n=15)	*26.7*	*26.7*	*13.3*
Female (n=8)	*37.5*	*37.5*	*12.5*
Male (n=7)	*14.3*	*14.3*	*14.3*

*mean z-score ± S.D; CDC=Centers for Disease Control and Prevention; WAZ=weight-for-age-z-score; HAZ-height-for-age-z-score; WHZ=weight-for-height-z-score; S.D.=standard deviation

6-2a Appendix B: Demographic and Anthropometric Description (WHO 2006 Child Growth Standards) of the 4-stool Cohort Population† (n=23)

Age (months)	58.7 ± 13.9
Females	N=13
Males	N=10

Anthropometrics	WAZ*	HAZ*	WHZ*
	WHO 2006	*WHO 2006*	*WHO 2006*
Ages			
<47.9 months (n=5)	-0.76±0.90	-2.21±0.65	0.76±0.88
Female (n=3)	-0.61±0.75	-2.17±0.81	0.95±0.42
Male (n=2)	-0.99±1.38	-2.29±0.60	0.49±1.58
48-59.9 months (n=8)	-1.54±0.76	-2.45±0.59	-0.05±0.87
Female (n=5)	-1.81±0.49	-2.57±0.38	-0.32±0.88
Male (n=3)	-1.09±1.04	-2.24±0.91	0.41±0.78
≥ 60 months (n=10)	-1.22±0.87	-1.75±1.22	-0.08±0.76**
Female (n=5)	-1.51±0.51	-1.77±1.17	-0.53±0.58
Male (n=5)	-0.93±1.11	-1.74±1.42	0.38±0.67
	% Global Malnutrition (WAZ <-2 S.D.)	*% Stunting (HAZ* <-2 S.D.)	*% Wasting (WHZ* <-2 S.D.)
Ages	*WHO 2006*	*WHO 2006*	*WHO 2006*
<47.9 months (n=5)	*0.0*	*40.0*	*0.0*
Female (n=3)	*0.0*	*33.3*	*0.0*
Male (n=2)	*0.0*	*50.0*	*0.0*
48-59.9 months (n=8)	*12.5*	*87.5*	*0.0*
Female (n=5)	*20.0*	*100.0*	*0.0*
Male (n=3)	*0.0*	*66.7*	*0.0*
≥ 60 months (n=10)	*20.0*	*30.0*	*0.0*
Female (n=5)	*20.0*	*40.0*	*0.0*
Male (n=5)	*20.0*	*20.0*	*0.0*

†These subjects provided the requisite 2 stool samples/week during week 0 and week 4
*mean z-score ± S.D.;** mean z-scores of body mass index (BMI) for age; WHO=World Health Organization; WAZ=weight-for-age-z-score; HAZ-height-for-age-z-score; WHZ=weight-for-height-z-score; S.D.=standard deviation

6-2b Appendix B: Demographic and Anthropometric Description (CDC 2000 Child Growth Standards) of the 4-stool Cohort Population† (n=23)

Age (months)	58.7 ± 13.9
Females	N=13
Males	N=10

Anthropometrics	WAZ*	HAZ*	WHZ*
	CDC 2000	*CDC 2000*	*CDC 2000*
Ages			
<47.9 months (n=5)	-0.84±1.11	-1.88±0.71	0.44±1.00
Female (n=3)	-0.68±1.04	-1.77±0.87	0.65±0.64
Male (n=2)	-1.09±0.62	-2.03±0.64	0.13±1.70
48-59.9 months (n=8)	-1.70±0.89	-2.14±0.61	-0.38±0.95
Female (n=5)	-1.98±0.63	-2.24±0.45	-0.67±0.98
Male (n=3)	-1.24±1.22	-1.98±0.91	0.1±0.82
≥ 60 months (n=10)	-1.20±0.88	-1.59±1.14	-0.10±0.65
Female (n=5)	-1.58±0.79	-1.81±1.29	-0.47±0.24
Male (n=5)	-0.82±0.87	-1.36±1.08	0.27±0.75

	% Global Malnutrition (WAZ <-2 S.D.)*	*% Stunting (HAZ* <-2 S.D.)*	*% Wasting (WHZ* <-2 S.D.)*
Ages	*CDC 2000*	*CDC 2000*	*CDC 2000*
<47.9 months (n=5)	*20.0*	*40.0*	*0.0*
Female (n=3)	*0.0*	*33.3*	*0.0*
Male (n=2)	*50.0*	*50.0*	*0.0*
48-59.9 months (n=8)	*50.0*	*62.5*	*0.0*
Female (n=5)	*60.0*	*60.0*	*0.0*
Male (n=3)	*33.3*	*66.7*	*0.0*
≥ 60 months (n=10)	*20.0*	*30.0*	*0.0*
Female (n=5)	*40.0*	*40.0*	*0.0*
Male (n=5)	*0.0*	*20.0*	*0.0*

†These subjects provided the requisite 2 stool samples per week during week 0 and week 4; *mean z-score ± S.D.;CDC=Centers for Disease Control and Prevention; WAZ=weight-for-age-z-score; HAZ-height-for-age-z-score; WHZ=weight-for-height-z-score; S.D.=standard deviation.

6-3 Appendix C: *Giardia* Prevalence Rates of Daycare Centers Surrounding Quetzaltenango, Guatemala

Daycare Center	Total Number of Subjects	Detected *Giardia* Prevalence Rate	Extrapolated *Giardia* Prevalence Rate*
2009 data (1 stool sample collected per week)			
Santa Ines	20	5.0%	6.5%
Casa Del Nino	19	10.5%	13.6%
San Martin Chiquito 2	10	20.0%	25.8%
Tojmech	19	26.3%	34.0%
San Martin Chiquito 1	23	30.4%	39.3%
Hogar Temporal Quetzaltenango	28	35.7%	46.1%
Hogar Comunitario Aldea La Estancia	34	44.1%	57.0%
Tojcon	19	47.4%	61.2%
2010 data (attempted 2 stool samples collected per week)			
La Esperanza	20	35%	N/A
San Mateo	16	37.5%	N/A
Duraznales	12	66.7%	N/A

*Results from the stool sample comparison indicate that *Giardia* prevalence rates based on a single stool sample only captured 77% of true *Giardia* cases, the 2009 prevalence have been adjusted accordingly for the extrapolated data.