PREVALENCE AND GENOTYPIC CHARACTERIZATION OF *GIARDIA DUODENALIS* ISOLATES AND ASSOCIATED RISK FACTORS OF INFECTION FROM ASYMPTOMATIC SCHOOL-GOING CHILDREN IN CHAWAMA, LUSAKA DISTRICT, ZAMBIA

BY

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UNIVERSITY OF ZAMBIA

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DECLARATION

I, **Shadreck Tembo**, do hereby declare that the work presented in this dissertation is the original work done by me. It is being submitted for the Degree of Master of Science in Medical Parasitology at the University of Zambia, Lusaka. This work has not been submitted for any degree at this or any other University.

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ABSTRACT

Giardia duodenalis (synonyms: *G. lamblia*, *G. intestinalis*) is a mammalian infective protozoan flagellate which infects humans. The genus *Giardia* consists of six species, namely *G. agilis, G. ardeae,* G. *psittaci, G. microti, G. muris* and *G. duodenalis*. Among these species, only *G. duodenalis* is known to infect several hosts, including humans. *G. duodenalis* is said to be a species complex comprising of eight genetic assemblages (A to H) and infection in humans being mainly caused by assemblages A and B. The purpose of this study was to determine prevalence and genotypic characterization of *Giardia duodenalis* isolates and associated risk factors of infection from asymptomatic school-going children in Chawama, Lusaka, Zambia.

A cross-sectional study was conducted between May and September 2017. A total of 329 faecal samples were collected from school-going children in Chawama within Lusaka City. *G. duodenalis* isolates were detected in asymptomatic school-going children aged three to sixteen years. All microscopically positive faecal samples were analyzed by semi-nested polymerase chain reaction (PCR) targeting the glutamate dehydrogenase (*gdh*) gene. Genotyping of amplified PCR products were further analyzed by restriction fragment length polymorphism (RFLP) and DNA sequence analysis.

The microscopic examination of faecal samples showed 10% (33/329; 95% CI = [0.07 – 0.13]) prevalence of *G. duodenalis* infection. The infection amongst girls was 12% (22/183; 95% CI = 5 – 61) and 7.5% (11/146; 95% CI = [0.39 – 0.49]) amongst boys. *Giardia* infections were more observed in children enrolled in public schools (15.4%, 19/123; 95% CI = [0.32 – 0.43]) than those in community schools (6.8%, 14/206; 95% CI = [0.57 – 0.68]) and infection was significantly ($p = 0.011$) associated with the type of school. The PCR-RFLP analysis revealed assemblages A and B in 27.3% (9/33; 95% CI = [0.14 – 0.46]) and 72.7% (24/33; 95% CI = [0.54 – 0.86), respectively. Furthermore, analysis with restriction enzymes identified sub-assemblages AII (27.3%, 9/33; 95% CI = [0.14 – 0.46]), BIII (12.1%, 4/33; 95% CI = [0.04 – 0.29), BIV (51.5%, 17/33; 95% CI= [0.34 – 0.69) and mixed BIII/BIV (9.1%, 3/33; 95% CI = [0.03 – 0.26]) infections. Assemblage B and sub-assemblage BIV infection was predominant in this study. However, no statistical significance ($p > 0.05$) was found between *G.duodenalis* assemblages/sub-assemblages and sex or age. The phylogenetic analysis showed the clustering of 27.6% (8/29; CI = [0.14 – 0.48]) and 72.4% (21/29; CI = [0.52 – 0.86]) of Zambian *Giardia gdh* gene sequences into assemblages A and B, respectively.

This study provides the first report on genotypic characterization of *G. duodenalis* infection in children in Chawama, Lusaka District, Zambia. The study revealed the circulation of assemblages A and B infection, which were further split into sub-assemblages AII, BIII and BIV. Mixed infections were observed between BIII/BIV. The assemblages detected could suggest anthroponotic transmission. The prevalence of *Giardia* infection was lower compared to previous surveys done in Zambia. *Giardia* infection was significantly associated with the type of school that children attended. Therefore, control measures aimed at improving hygiene in schools and the communities, along with education campaigns could help in curbing giardiasis.

*Keywords***:** *Giardia duodenalis*; Giardiasis; Genotyping; Glutamate dehydrogenase; Phylogenetic analysis; Zambia

DEDICATION

I dedicate this dissertation to my wife Charity and our three daughters; Taonga, Tabo and Tamanda for the moral and spiritual support rendered to me.

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LIST OF ABBREVIATIONS

Bg	βeta – giardin gene
BLAST	Basic Local Alignment Search Tool
DEBS	District Education Board Secretary
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine Tetra-acetic Acid
ELISA	Enzyme-Linked Immuno-sorbent Assay
GDH	Glutamate Dehydrogenase gene
IDA	Iron deficiency Anaemia
IFA	Immunofluorescence Antigen
IPIs	Intestinal Parasitic Infections
MoH	Ministry of Health
PCR	Polymerase Chain Reaction
RDTs	Rapid Diagnostic Tests
RFLP	Restriction Fragment Length Polymorphism
SSU rRNA	small subunit ribosomal ribonucleic Acid
TPI	Triose Phosphate Isomerase
UNZABREC	University of Zambia Biomedical Research Ethics Committee
USA	United States of America
UTH	University Teaching Hospital
VAD	Vitamin A Deficiency
WGO	World Gastroenterology Organization
WHO	World Health Organization

CHAPTER 1: INTRODUCTION

1.1 Background

Giardia duodenalis (synonyms: *G. lamblia*, *G. intestinalis*) is a mammalian infective protozoan flagellate which accounts for 250 – 300 million symptomatic human infections annually (Ankarklev *et al*., 2010; Feng and Xiao, 2011; Einarsson *et al*., 2016). It is a major cause of gastrointestinal infections in humans (Zumla *et al.*, 2003; Alum *et al*., 2012), with infants, young children, and young adults being greatly at risk of infection (Torgerson *et al*., 2015). The infection caused is associated with about 2.5 million deaths that occur in developing countries per year (Gelanew *et al*., 2007; Alum *et al*., 2012).

Giardia infection is prevalent in both the developed and developing countries of the world, with higher rates of infection being reported in the latter (Alum *et al*., 2012). In developing countries, giardiasis is common among the low-income families who are prone to poor sanitary environments and housing infrastructure (Mascarini *et al*., 2006; Tashima *et al*., 2009). The infection is transmitted through direct faecal-oral route or indirectly by ingesting *Giardia* cysts in contaminated food or water (Thompson, 2000; Savioli *et al*., 2006). It affects all age groups, with higher rates of infection being reported in children who are exposed to poor hygiene and sanitation (Savioli *et al*., 2006; Buret and Cotton, 2011; Feng and Xiao 2011). The infection presents with various clinical symptoms such as asymptomatic cyst carriers, acute diarrhoea or chronic disease associated with weight loss and growth retardation in children (Ankarklev *et al*., 2010; Kotloff *et al* 2013; Certad *et al*., 2017). Complications have also been observed in children with chronic giardiasis and these include failure to thrive and cognitive impairment (Carvalho-Costa et *al*., 2007; Nematian et *al*., 2008; Quihui *et al*., 2010).

Giardiasis is a common disease in livestock (dairy cattle) and companion animals (cats and dogs); thus, it is of veterinary health significance (Feng and Xiao, 2011). The infection is most common in calves (O'Handley *et al*., 2001) as some studies have shown that both dairy and beef calves may harbour more than one assemblage (genotype) of *G. duodenalis* (Trout *et al*., 2005; Mendonca *et al*., 2007). The infection is most prominent in calves aged between four days and three months old (Ralston *et al*., 2003). The infected calves shed more cysts in their faeces and this result in them becoming potential reservoirs of zoonotic infections (O'Handley *et al*., 1999). As such, giardiasis contributes to significant morbidity and mortality in calves (Appelbee *et al*., 2005; Hunter and Thompson, 2005; Savioli *et al*., 2006).

Giardia duodenalis can be classified on the basis of host of origin, morphology and molecular characterisation (Cacciò *et al*., 2018). Further, analysis of *Giardia* is based on intrinsic characteristics of the parasite which include antigenic factors and isoenzymes (Adam, 2001; Feng and Xiao, 2011). Genotyping studies have shown that *G. duodenalis* comprises of eight assemblages designated A to H (Caccio *et al*., 2005; Monis *et al*., 2009; Ryan and Cacciò, 2013). Assemblages A and B being considered zoonotic as they infect both humans and other mammals (Gelanew *et al*. 2007; Feng Y, Xiao, 2011; Cacciò *et al*., 2018), whilst assemblages C to H are considered to be host-restricted (Feng and Xiao, 2011; Cacciò *et al*., 2018).

The diagnosis of giardiasis in humans is mainly based on signs, clinical symptoms and microscopic detection of cysts or trophozoites (Yakoob *et al*., 2005; Hawash, 2014). Several diagnostic techniques like microscopy, immunoassays and molecular methods can be used to diagnose *Giardia* infection (Babaei *et al*., 2011; Koehler *et al*., 2014). The polymerase chain

reaction (PCR), restriction fragment length polymorphism (RFLP) and DNA sequencing assays are some of the molecular assays commonly used to genotype *Giardia* and they work by targeting specific genetic markers (Wielinga *et al*., 2007).

1.2 Statement of the problem

Giardia infection is an emerging public health problem around the world and accounts for about 2.5 million diarrhoea-associated deaths (Adam, 2001). Despite being commonly isolated in children with diarrhoea, the disease is considered neglected (WGO, 2012; Einarsson *et al*., 2016). The infection is highly prevalent in developing countries among people who are exposed to poor sanitary environments and housing infrastructure (Mascarini *et al*., 2006; Tashima *et al*., 2009). *Giardia* causes severe impact on growth and cognitive development in children living in developing countries and thus its inclusion in the world health organization's (WHO's) "Neglected Disease Initiative" (Savioli *et al*., 2006).

Giardia infections are often underreported in many countries as screening for intestinal parasites is rarely performed (Yoder *et al*., 2010). In Zambia, high infection rates of 29 – 53.8% of giardiasis have been reported in children (Graczky *et al*., 2005; Siwila *et al*., 2010) an indication that the parasites is circulating in the population. The infection ranks among the top five causes of diarrhoeal diseases in Zambian children, that is rotavirus at 67.6%, Adenovirus at 41.5%, Entero-toxigenic *Escherichia coli* at 40.7%, *Salmonella at* 38.4% and *Giardia* at 37% (Chisenga *et al*., 2018), respectively. Worse still, complications of cognitive impairment and failure to thrive in children with chronic giardiasis have been reported (Al-Mekhlafi *et al*., 2010; Al-Mekhlafi *et al*., 2013).

Giardia duodenalis is a species complex that infects humans and other vertebrate hosts such as livestock (cattle, sheep goats, pigs) and companion animals like dogs and cats (Adam, 2001; Becher *et al*., 2004; Lasek-Nesselquist *et al*., 2010; Farzan *et al*., 2011; Bouzid *et al*., 2015; Akinkuotu *et al*., 2016; Wang *et al*., 2016). The infection is frequently observed in dairy calves (O'Handley *et al*., 2000; Thompson, 2000; Ralston *et al*., 2003) through shedding of more cysts in their faeces and this make them to be potential reservoirs of zoonotic infections (O'Handley *et al*., 1999; Khan *et al*., 2011; Malekifard and Ahmadpour, 2018). For instance, some previous studies have demonstrated the zoonotic significance of both dairy and beef calves in harbouring more than one assemblage (genotype) of *G. duodenalis* infection (Trout *et al*., 2005; Mendonca *et al*., 2007). Further, previous studies have demonstrated sub-assemblage AI and host-restricted assemblage E of *G. duodenalis* infection in humans from animals (Olson *et al*., 2004; Feng and Xiao, 2011; Du *et al*., 2015; Qi *et al*., 2015; Ankarklev *et al*., 2018). However, the assemblages/sub-assemblages circulating and infecting humans in Zambia are unknown as no molecular studies have been conducted.

Giardiasis is common in all age groups, but children in urban unplanned settlements are at highest risk of acquiring infection (Mbae *et al*., 2016). Several unplanned settlements exist within the city of Lusaka in Zambia. These unplanned settlements are characterized with poor sanitary environments, overcrowding, low socio-economic status and erratic water supply (Ndhlema, 2000). One of the unplanned settlements includes Chawama the study site in this study. Its population is at higher risk of acquiring waterborne or foodborne infections due to *Cholera* and *Giardia*, respectively. In fact, *Cholera* outbreaks have previously being reported in

Chawama and including other townships within the city of Lusaka (Rohmawati, 2010; WHO, 2010; Sinyange *et al*., 2018) an indication of poor sanitary conditions.

1.3 Study Justification

Molecular studies in Africa have demonstrated the circulation of assemblages or sub-assemblages infecting humans (Squire and Ryan, 2017). In Zambia, there is a knowledge gap and thus the reason of conducting this molecular study to know which assemblages or sub-assemblages are in circulation and infecting the children. Genotypic characterization of *G. duodenalis* has been reported to be a useful method in epidemiological surveys or outbreak investigations (Robertson *et al*., 2006). Knowing and understanding the assemblages or sub-assemblages circulating in the population is very crucial in tracing sources of infection. The study also intends to identify risk factors associated with giardiasis among school-going children in Chawama, thereby coming up with evidenced based control measures. The information gathered would be helpful to various stakeholders at the Ministry of Health, Ministry of General Education, school authorities and the community on how to prevent human-human transmission.

1.4 Research Questions

1.4.1 What is the prevalence of *Giardia* infection in school-going children in Chawama?

1.4.2 Which assemblages or sub-assemblages of *Giardia* infect school-going children in Chawama?

1.4.3 What are the risk factors associated with *Giardia* infection among school-going children in Chawama?

1.5 Objectives

1.5.1 General objective

To determine prevalence and genotypic characterization of *Giardia duodenalis* isolates and associated risk factors of infection from asymptomatic school-going children in Chawama, Lusaka District, Zambia.

1.5.2 Specific objectives

1. To determine the prevalence of *Giardia duodenalis* infection among the school-going children in Chawama, Lusaka District.
2. To determine genetic assemblages of *Giardia duodenalis* by PCR-RFLP and DNA sequence analysis in school-going children in Chawama, Lusaka District.
3. To determine the risk factors of *Giardia duodenalis* infection among school-going children in Chawama, Lusaka District.

CHAPTER 2: LITERATURE REVIEW

2.1 *Giardia* parasite

Giardia is an intestinal protozoan flagellate (Filice, 1952; Erlandsen *et al*., 1990). It was first discovered by Leeuwenhoek in 1681 when examining his own stool under the microscope (Ortega and Adam, 1997). The parasite belongs to the genus *Giardia*, phylum Sarcomastigophora, class Zoomastigophora and a member of the order Diplomonadida (Adam, 2001; Gelanew *et al*., 2007). The genus *Giardia* consists of six species, namely *G. agilis* infecting amphibians, *G. ardeae* and *G. psittaci* infect birds, *G. microti* and *G. muris* infect rodents and *G. duodenalis* infects mammals (Olson *et al*., 2000; Thompson, 2002). Among these species, only *G. duodenalis* is known to infect several hosts, including humans (Thompson, 2004; Caccio and Ryan, 2008; Einarsson *et al*., 2016).

2.2 Morphology of *Giardia* species

Giardia species have two morphological stages, namely the trophozoite and the cyst (Ivanov, 2010). The trophozoite is pear shaped, bilaterally symmetrical, measuring about 10 – 20 μm long and 5 – 15 μm wide (Rajurkar *et al*., 2012). It has a convex dorsal surface with a large ventral adhesive (sucking) disc, two nuclei, four pairs of flagella and a pair of median body (Ivanov, 2010). On the other hand, the *Giardia* cyst is oval in shape, measuring about 8 – 12 μm long and 7 to 10 μm wide, with an outer membrane comprising of thin hyaline wall (Ivanov, 2010). An immature cyst has two nuclei in its early stages of development, and mature cyst has four nuclei with curved median bodies and linear axonemes (Ivanov, 2010). Thus, differentiation of *Giardia* species is based on trophozoite morphology (Thompson, 2002; Thompson, 2004) as shown in Table 2.1.

Table 2.1: *Giardia* species (Thompson, 2002)

Species	Host	Morphologic features	Length/width of the trophozoites (mm)
Giardia agilis	Amphibians	Long and narrow trophozoites with club-shaped median bodies.	20-30 / 4-5
Giardia ardeae	Birds	Rounded trophozoites, prominent notch in ventral disc and rudimentary caudal flagellum. Median bodies' round-oval to claw-shaped.	10 / 6
Giardia duodenalis	Humans, domestic and wild mammals	Pear-shaped trophozoites with claw-shaped median bodies.	12-15 / 6-8
Giardia microti	Rodents	Trophozoites similar to *G. duodenalis*. Cysts contain fully differentiated trophozoites.	12-15 / 6-8
Giardia muris	Rodents	Rounded trophozoites with small, round median bodies.	9-12 / 5-7
Giardia psittaci	Birds	Pear-shaped trophozoites, with no ventral-lateral flange. Claw-shaped median bodies.	14 / 6

2.3 Genetic assemblages and sub-assemblages

Giardia duodenalis exhibits a high degree of genetic diversity (Caccio *et al*., 2005; Monis *et al*., 2009). It is a species complex comprising of eight (A-H) genetic assemblages (Caccio and Ryan, 2008; Feng and Xiao, 2011; Ryan and Cacciò, 2013). The host range of these assemblages appear to vary widely (Caccio and Ryan, 2008; Minetti *et al*., 2016). Assemblages A and B infect humans and other mammals such as domestic animals, livestock and a number of wildlife species (Xiao and Fayer, 2008; Feng and Xıao, 2011; Ryan and Caccio, 2013; Minetti *et al*., 2016). Whilst, assemblages C – H are said to be host-restricted, assemblages C and D infect mainly Canids, assemblage E infects hooved animals, assemblage F infects cats, assemblage G

infects rats and assemblage H infects marine mammals (Feng and Xıao, 2011; Ryan and Caccio, 2013; Minetti *et al*., 2016; Cacciò *et al*., 2018). Other studies have reported host-restricted assemblage infection in human, such as assemblage C in China and Slovakia (Liu *et al*., 2014; Strkolcova *et al*., 2016), assemblage D in German (Broglia *et al*., 2013), assemblage E in Egypt, Australia and Brazil (Foronda *et al*., 2008; Helmy *et al*., 2014; Abdel-Moein and Saeed, 2016; Fantinatti *et al*., 2016; Scalia *et al*., 2016; Zahedi *et al*., 2017) and assemblage F in Ethiopia (Gelanew *et al*., 2007), respectively. An indication that host specificity of assemblages is not as rigid as previously thought because strains of *G. duodenalis* have undergone an evolutionary process of adaptation to other species hosts (Traub *et al*., 2009). Therefore, transmission of zoonotic and host-restricted assemblage infection is said to occur in environments where humans and animals live in close proximity to each other (Feng and Xiao, 2011; Ankarklev *et al*., 2018).

Further analysis shows that *Giardia* assemblages A and B can be divided into sub-assemblages AI, AII, AIII, AIV, BI, BII, BIII and BIV (Monis *et al*., 2003; Sprong *et al*., 2009). Molecular characterization of *G. duodenalis* isolates is achieved by targeting specific genetic markers (genes or loci) and these include glutamate dehydrogenase (*gdh*), small subunit ribosomal RNA (SSU *rRNA*), β- giardin (*βg*) and triose phosphate isomerase (*tpi*) gene (Lalle *et al*., 2005; Durigan *et al*., 2014; Karim *et al*., 2015; Minetti *et al*., 2015). Sub-assemblages AI, AIII and AIV infect only animals (Sprong *et al*., 2009; Ryan and Cacciò, 2013), while AII mainly causes anthroponotic infections (Caccio and Ryan, 2008; Yang *et al*., 2010; Sarkari *et al*., 2012). Sub-assemblages BI and BII have been associated with animal infection in monkeys and dogs (Monis *et al*., 2003). Sub-assemblages BIII and BIV infections have been reported in human and occasionally in animals (Sprong *et al*., 2009; Feng and Xiao, 2011; Ryan and Cacciò, 2013).

Mixed infections involving assemblages or sub-assemblages of *Giardia* have been reported in humans (Gelanew *et al*., 2007; Feng and Xiao, 2011). Globally, the prevalence of mixed infections ranges from 2.0% to 21.0% in humans (Amar *et al*., 2002; Lalle *et al*., 2005; Al-Mohammed, 2011). Their occurrence could be as a result of genetic exchanges between assemblage A and B isolates in human faeces (Teodorovic *et al*., 2007) or human exposure to multiple sources of infection such as consumption of cysts in contaminated food or water and human-human transmission (Damitie *et al*., 2018). The detection of mixed infections makes it difficult to clearly link assemblages/sub-assemblages to clinical symptoms (Feng and Xiao, 2011; Jerez-Puebla *et al*., 2015).

Giardia duodenalis is known for cross-species transmission of zoonotic infections (Thompson, 2004; Sprong *et al*., 2009). Transmission of zoonotic associated assemblages is common where there is ecological overlap between species (Hunter and Thompson, 2005; Lalle *et al*., 2005; Foronda *et al*., 2008; Goldberg *et al*., 2008). The occurrence of same species assemblages in human and non-human hosts such as dogs, cats, livestock and wildlife have been reported (Thompson and Smith, 2011). As such, these animals act as potential reservoirs of zoonotic infection (Traub *et al*., 2004; Feng and Xiao, 2011; Julio *et al*., 2012).

Globally, the distribution of *Giardia* assemblages A and B varies from one country to another (Feng and Xiao, 2011). Some studies conducted in the USA, Canada, Korea and Uganda reported only assemblage A infection (Caccio *et al*., 2005), whilst other studies in Ghana and India reported only assemblage B infection (Tak *et al*., 2014; Anim-Baidoo *et al*., 2016), respectively. Other studies conducted in the Netherlands, Albania, Brazil, Slovenia, Morocco and

Kenya reported both assemblages A and B infection (Caccio *et al*., 2005; Berrilli *et al*., 2006; El Fatni *et al*., 2014; Soba *et al*., 2015; Mbae *et al*., 2016). Assemblage B has been shown to be most common than assemblage A (Caccio *et al*., 2008). For instance, analysis of 2800 *G. duodenalis* isolates from humans revealed predominance of assemblage B at 58%, followed by assemblage A at 37% and 5% for mixed infections (Ryan and Caccio, 2013). The distribution of assemblages and sub-assemblages infection has been shown in Table 2.2 below.

Table 2.2: Distribution of *G. duodenalis* assemblages

Assemblage (%)	**Sub-assemblage**	**Host**	**Distribution**	**Reference**
A(100)	-	humans	United States	van Keulen, *et al*., 2002
A (33.0) B (64.0) A/B (3.0)	-	humans	England	Minetti *et al*., 2015
A (35) B (65)	-	humans	Netherlands	van Der Giessen *et al*., 2007
A (52.7) B (47.3)	AII (40.6) BIII (21.9) BIV (12.5) BIII/BIV (25.0)	Humans	Brazil	Faria *et al*., 2016
A (18.1) B (81.8)	AII (18.1) BIII (9.08) BIV (72.7)	humans	Morocco	El Fatni *et al*., 2014
A (5.0) B (80.0) E (15.0)	-	Humans	Egypt	Foronda *et al*., 2008
B (100)	BIII (100)	humans	Ghana	Anim-Baidoo *et al*., 2016
A (1.4) B (88.9) A/B (9.7)	AII/ BIII (4.0) BIII (12.0) BIV (28.0) BIII/BIV (56.0)	humans	Kenya	Mbae *et al*., 2016
A (100)	AII (100)	humans	Central African Republic	Sak *et al*., 2013

2.4 Epidemiology of *Giardia* infection

Giardia duodenalis infection is the most common intestinal infection of humans and other vertebrates (Adam, 2001; Garcia, 2005; Feng and Xiao, 2011). Globally, *Giardia* infects about 200 million people in Asia, Africa, and Latin America (Yason and Rivera, 2007; Feng and Xiao, 2011), including the United States and United Kingdom (Noor *et al.*, 2007). Every year, about 500, 000 new cases of giardiasis are reported globally (Kosek *et al*., 2003). It is estimated that 3000 million people in developing countries live in poor sanitary environments (Upcroft and Upcroft, 2002). *Giardia* causes about 2.5 million diarrhoea-associated deaths per year world widely (Upcroft and Upcroft, 2002). Its distribution varies in both developed and developing countries with most infections being common in developing countries (Eligio-Garcia *et al*., 2008; Cacciò and Sprong, 2014). As such, *G. duodenalis* was included in the "Neglected Disease Initiative" of the World Health Organization (WHO) in 2004 because of its burden and impact on socio-economic development in developing countries (WHO, 2004; Savioli *et al*., 2006). Furthermore, majority of reported *Giardia* outbreaks have been caused by waterborne (74.8%), followed by foodborne (15.7%), human to human (2.5%) and animal contact (1.2%) transmission (Adam *et al*., 2016). In humans, *Giardia* infection is at its peak in children aged 0 - 9 years and adults aged 45 - 49 years (Painter *et al*., 2015). The prevalence of infection can also be influenced by the prevailing season, such that high infection rates have been reported during summer months (Cacciò and Sprong, 2014).

2.5 Prevalence of *G. duodenalis* infection

Giardia infection is prevalent worldwide amongst the infants, young children, adults, international adoptees, travelers (Johnston *et al*., 2010; Feng and Xiao, 2011; Al-Mekhlafi *et al*.,

2013; Durigan *et al*., 2014; Takaoka *et al*., 2016). The infection is also common among day- care centre workers, diaper-age children attending care centers, orphanages, home sexual and dairy farm workers (Beltrami *et al*., 2005; Mascarini and Donalisio, 2006; Gelanew *et al*., 2007; Khan *et al*., 2010; Mark-Carew *et al*; 2010; Duffy *et al*., 2013). The prevalence of giardiasis ranges from 0.1% to 5% in developed countries (Smith *et al*., 2006; Minetti *et al*., 2015; Zylberberg *et al*., 2017) and as high as 15 – 55% in developing countries (Yakoob *et al*., 2005; Al-Saeed and Issa, 2006; Alum *et al*., 2012). As such, high prevalence rates have been reported in many African countries including Zambia (Graczyk *et al*., 2005; Siwila *et al*., 2011; Squire and Ryan, 2017). Find below is Table 2.3 showing the distribution of giardiasis in humans around the world.

Table 2.3: Prevalence of *G. duodenalis* infection

Prevalence (%)	Host	Country	Reference
1.4	Humans	United States	Church *et al*., 2010
4.0	Humans	Belgium	Geurden *et al*., 2009
1.5	Humans	Germany	Sagebiel *et al*., 2009
1.6 and 7.6	Humans	Australia	Hellard *et al*., 2000; Read *et al*., 2002
8.9	Humans	Brazil	Colli *et al*., 2015
11.7	Humans	Argentina	Molina *et al*., 2011
5.0	Humans	India	Chanu *et al*., 2018
28.1	Humans	Malaysia	Al-Mekhlafi, 2017
11.3	Humans	Egypt	Naguib *et al*., 2018
12.5	Humans	Morocco	El Fatni *et al*., 2014
4.6 and11.1	Humans	Kenya	Wasike *et al*., 2015; Mbae *et al*., 2016
7.75 and 9.9	Humans	South Africa	Nxasana *et al*., 2013; Samie *et al*., 2014
29 and 53.8	Humans	Zambia	Graczyk *et al*., 2005; Siwila *et al*., 2010

2.6 Transmission of *Giardia* infection

Giardiasis is transmitted through direct faecal-oral route (person-person, animal-human) or indirectly through ingestion of infectious cysts in contaminated food and water , with the indirect route being the major route of transmission (Thompson, 2000; Savioli *et al*., 2006; Ryan and Caccio, 2013; Boarato-David *et al*., 2017; Ryan *et al*., 2019). Transmission of human - human infection is common among people of low socio-economic status who are exposed to poor sanitary environments, unsafe drinking water sources and practicing poor personal hygiene (Savioli *et al*., 2006; Samie *et al*., 2009; Ankarklev *et al*., 2010; Fletcher *et al*., 2012). The transmission of human – human infection is common among the overcrowded families (Balcioglu *et al*., 2003), children in day-care centres (Savioli *et al*., 2006; Duffy, 2013), pre-school and primary school-going children (Perez-Cordon *et al*., 2008; Ratanapo *et al*., 2008; Al-Saeed and Issa, 2010) and children in orphanages (Bailey *et al*., 2013), respectively.

The consumption of contaminated food or water with *Giardia* cysts can aid the transmission of giardiasis indirectly (Thompson, 2000; Baldursson and Karanis, 2011; Boarato-David et *al*., 2017). Thus, infected food handlers shedding *Giardia* cysts in their faeces or individuals involved in changing diapers from infected individuals can transmit foodborne infections when their personal hygiene standards have being compromised (Smith *et al*., 2006; Hoffmann *et al*., 2007; Boarato-David et *al*., 2017). As such, foodborne outbreaks have been reported in different parts of the world (Smith *et al*., 2006; Blasi *et al*., 2008; Yoder *et al*., 2010 Painter *et al*., 2015). Further, waterborne outbreaks have been reported in the United States, the United Kingdom, Norway, Finland and Asia mainly due to consumption of untreated or poorly treated water (Nygård *et al*., 2006; Eisenstein *et al*., 2008; Daly *et al*., 2010; Rimhanen-Finne *et al*., 2010;

Painter *et al*., 2015). An estimated 100 waterborne outbreaks occurred during the year 2004 to 2010 (Karanis *et al*., 2007; Baldursson and Karanis, 2011). The largest one occurred in Bergen (Norway) in 2004 and affected about 1500 people (Nygård *et al*., 2006; Robertson *et al*., 2006**)**.

Transmission zoonotic infections have been reported, especially from companion animals like dogs and cats to humans (Thompson, 2004; Traub *et al*., 2009; Bouzid *et al*., 2015). For instance, transmission of sub-assemblage AI (zoonotic) infection from a dog to a child who lived in the same household was reported in a Brazilian study (Volotao *et al*., 2007). Further, transmission of host-restricted assemblages C, D, E, and F from animals to humans have been reported in Australia, German, China, Brazil Egypt and Ethiopia (Gelanew *et al*., 2007; Foronda *et al*., 2008; Broglia *et al*., 2013; Helmy *et al*., 2014; Liu *et al*., 2014; Abdel-Moein and Saeed, 2016; Fantinatti *et al*., 2016; Scalia *et al*., 2016; Strkolcova *et al*., 2016; Zahedi *et al*., 2017), respectively.

2.7 *Giardia* life cycle

Giardia parasite has a simple direct life cycle alternating between the rapidly multiplying trophozoite and the infective cyst (Anuar *et al*., 2014; Einarsson *et al*., 2016). Cysts are the infective stage of *Giardia* that cause giardiasis, whilst trophozoites (vegetative form) are the replicative stage of the parasite (Adam, 2001). *Giardia* infection is initiated by ingestion of infective cysts through direct faecal-oral or indirectly through consumption of contaminated food or water in which cysts are stimulated to excyst in the stomach due to prevailing acidic conditions to become trophozoites (Adam, 2001; Gardner and Hill, 2001; Ankarklev *et al*., 2010). The released trophozoites undergo multiple mitotic divisions (Feng and Xiao, 2011) and

reproduce asexually by simple binary fission (Poxleitner *et al*., 2008). The emerging trophozoites migrate to the duodenum and upper part of the small intestines by attaching to the intestinal walls being facilitated by the adhesive disc, as a result infection become established in these sites due to favourable alkaline conditions (Dawson, 2010). The adhesive disc facilitates attachment to the intestinal walls and also acts as the main virulence factor for *Giardia* (Dawson, 2010). The trophozoites migrate further to the colon where encystation takes place and cysts are discharged in faeces (Thompson, 2000; Huang and White, 2006). *Giardia* cysts become instantly infectious upon their release in faeces and remain potentially infectious for several months as they have ability to withstand unfavourable environmental conditions (Feng and Xiao, 2011; Duffy *et al*., 2013). Shown below in figure 2.1 is the life cycle of *G. duodenalis*.

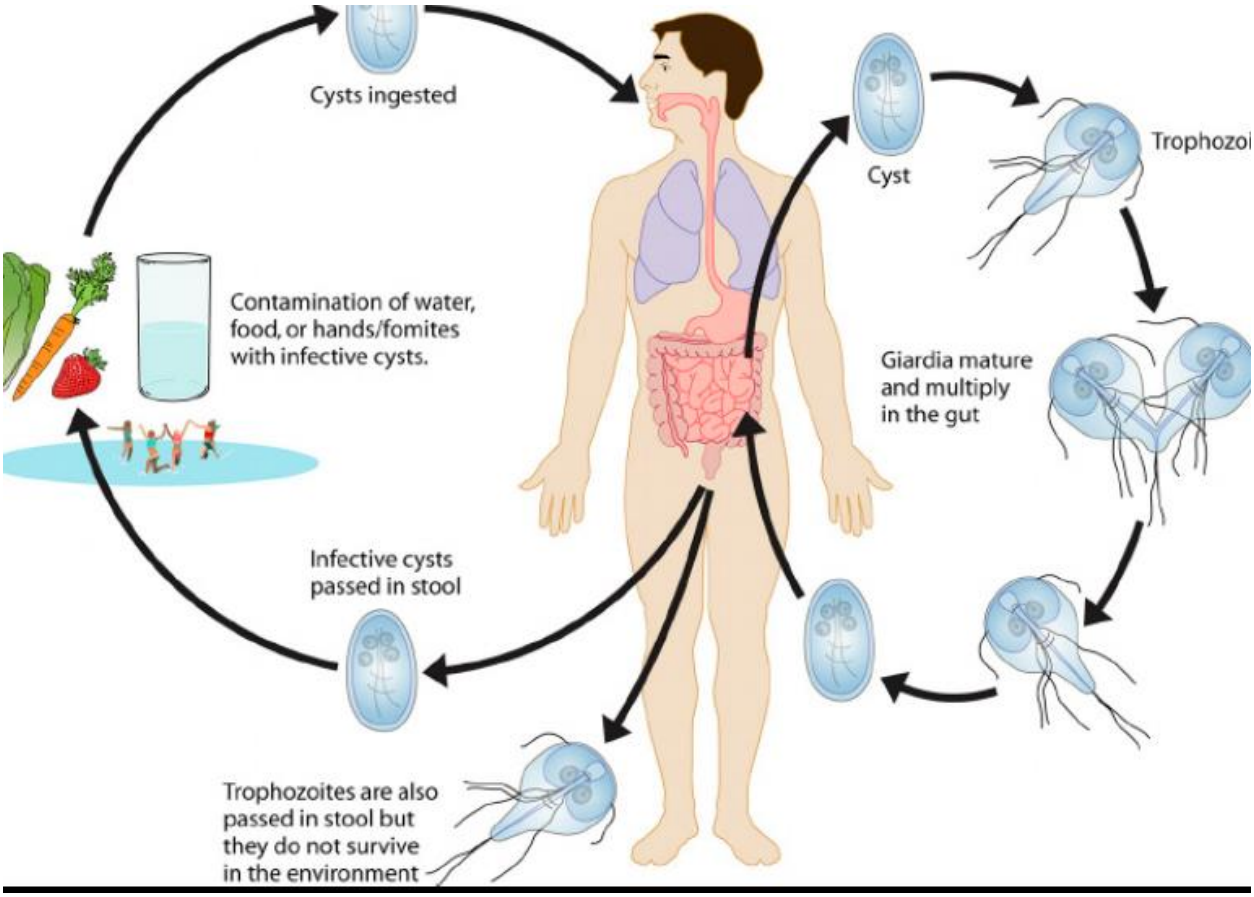

Figure 2.1: Life cycle of *G. duodenalis* (Esch and Petersen, 2013)

2.8 Giardiasis

Giardia duodenalis causes giardiasis (*Giardia* infection) in humans (Adam, 2001; Ismail *et al*., 2016). Globally, an estimated 1.7 billion cases of diarrhoeal diseases occur per annum have been recorded (WHO, 2014). The major causes of diarrhoeal infections in humans include parasites (*G. duodenalis*, *Cryptosporidium parvum* and *Entamoeba histolytica*), bacteria (*Salmonella* species, *Shigella* species and Enter-toxigenic *Escherichia coli*) and rotavirus (Svennerholm and Tobias, 2008; Walker, 2015; Parashar *et al*., 2016; Riddle *et al*., 2016). *Giardia* infection is reported worldwide in about 280 million people every year (Ryan and Cacciò, 2013; Einarsson *et al*., 2016; Squire and Ryan, 2017), with the infection ranking among the top five aetiological agents of diarrhoeal disease in Zambia; Rotavirus (67.6%), *Adenovirus* (41.5%), Entero-toxigenic *Escherichia coli* (40.7%), *Salmonella* (38.4%) *and* 37% for *G. duodenalis* (Chisenga *et al*., 2018).

2.8.1 Predisposing factors

The predisposing factors of *Giardia* infection include poor sanitary environments, lack of good toilet facilities, overcrowding and low socio-economic status among people practicing low standards of hygiene (Stephenson *et al*., 2000; Saksirisampant *et al*., 2003; Feng and Xiao, 2011). Other factors include lowered immunity and malnourishment in young aged children (Castro *et al*., 2004; Al-Mekhlafi *et al*., 2005), nursing infected children or children attending day care centres because of compromised personal hygiene (Ratanapo *et al*., 2008; Espelage *et al*., 2010), storage of water in previously contaminated containers by households not directly supplied with piped water (Cifuentes *et al*., 2004) and non-exposure to parasitic infections by travelers to endemic areas (Stuart *et al*., 2003). Further, males are at risk when playing in

contaminated environments during outdoor recreational activities (Mohammed Mahdy *et al*., 2009; Espelage *et al*., 2010) and females when engaged in indoor activities that include changing diapers or washing nappies from infected children (Tashima *et al*., 2009; Mohammed Mahdy *et al*., 2009; Ogbuu *et al*., 2018), respectively.

2.8.2 Pathogenesis of *G. duodenalis* infection

Giardia parasite is non-invasive, attaches and replicates within the intestinal epithelium (Bartelt and Sartor, 2015). The trophozoites attach to the brush border surface of the upper part of the small intestine causing shortening of the epithelial microvilli (Cotton *et al*., 2011). This attachment causes increased enterocyte apoptosis, disturbance of the intestinal barrier function, proliferation of host lymphocytes, CD8+ lymphocyte-mediated shortening of brush border microvilli, (villous atrophy), small intestinal malabsorption, disaccharidase deficiencies, anion hypersecretion and increased intestinal transit rates (O'Handley *et al*., 2001; Chin *et al*., 2002; Scott *et al*., 2002; Troeger *et al*., 2007; Koot *et al*., 2009). The disturbance to the intestinal barrier function causes disruption of F-actin, zonula occluding - 1 (ZO-1), claudin-1 and claudin-4, occludin and α-actinin which regulates epithelial permeability (Teoh *et al*., 2000; Chin *et al*., 2002; Scott *et al*., 2002; Troeger *et al*., 2007; Halliez *et al*., 2014). Thus, there is loss of epithelial barrier integrity due to activation of epithelial myosin light chain kinase which causes destruction of epithelial tight junctional proteins (Chin *et al*., 2002; Chin *et al*., 2006; O'Hara and Buret, 2008; Chin *et al*., 2003; Buret and Bhargava, 2013). Furthermore, the release of cysteine proteases causes proteolytic disruption of epithelial villin, which is an important constituent of brush border microvilli (Rodriguez-Fuentes *et al*., 2006; Bhargava *et al*., 2013). As a result, there is increased permeability of enterocytes and fluid secretion, impaired absorption

of glucose, sodium, chloride and diarrhoea (Cotton *et al*., 2011; Bartelt and Sartor, 2015). The pathogenic effects of *Giardia* may be compounded by the degradation of local mucins and by the parasite itself (Amat *et al*., 2015) thereby causing the translocation of commensal bacteria through the epithelium (Beatty *et al*., 2013; Chen *et al*., 2013; Halliez *et al*., 2014; Amat *et al*., 2015). The severity of disease and apoptosis are dependent on the virulent factors of the parasite, immunological status of the host, infection dose and nature of intestinal microflora present (Chin *et al.*, 2002; Scott *et al.*, 2002; Pierce and Kirkpatrick 2009; Tungtrongchitr *et al*., 2010; Feng and Xiao, 2011). The toxins and metabolic products produced by *Giardia* assemblages have also been associated with pathogenesis, but the mechanisms involved are poorly understood (Tungtrongchitr *et al*., 2010; Feng and Xiao, 2011; Buret *et al*., 2015).

2.8.3 Clinical symptoms

Giardiasis symptoms can either be acute or chronic diarrhoea, weight loss and malabsorption; however the majority of cases are asymptomatic (Ankarklev *et al*., 2010; Certad *et al*., 2017). Several factors play a role in the manifestation of clinical symptoms and these include host response, infection dose, *Giardia* species and assemblages (Pierce and Kirkpatrick, 2009). In addition, outcome of symptoms are dependent on parasite factors which include rate of multiplication, expression of variable surface proteins and ability to invade immune response (Robertson *et al*., 2010). *Giardia* symptoms usually appear in about 1 to 2 weeks after infection (Buret *et al*., 2015). The common symptoms in acute infection include foul-smelling diarrhoea, extensive flatulence, bad taste, colics, nausea, anorexia, epigastric pain and sometimes the infection is self-limiting (Chakarova, 2004; Feng and Xiao, 2011; Einarsson *et al*., 2016). Chronic giardiasis is mainly associated with lethargy, headache, muscle pain, diarrhoea,

progressive weight loss, loss of appetite and malabsorption (Squire and Ryan, 2017). Further, chronic giardiasis has been associated with complications of long-term growth retardation, protein-energy malnutrition (PEM), vitamin A deficiency (VAD), iron deficiency anaemia (IDA), cognitive impairment in children and post-infectious irritable bowel syndrome (Gwee, 2005; Al-Mekhlafi *et al*., 2010; Cotton *et al*., 2011; Al-Mekhlafi *et al*., 2013; Squire and Ryan, 2017). Asymptomatic infections are most common and they are diagnosed through microscopic detection of *Giardia* cysts in faecal samples (Hooshyar *et al*., 2019). The non-detection of infection in asymptomatic carriers poses a serious public health risk to the uninfected individuals (Anim-Baidoo *et al*., 2016; Oliveira-Arbex *et al*., 2016). As such, they serve as potential reservoirs of infection due to continuous shedding *Giardia* cysts in their faeces (Anim-Baidoo *et al*., 2016).

Giardia assemblages infecting humans are presented with various clinical symptoms (Homan and Mank, 2001; Caccio and Ryan, 2008). The symptoms appear to be assemblage-dependent (Haque et *al*., 2005; Puebla *et al*., 2014; Pestechian *et al*., 2014). For example, some previous studies found assemblage A infection in individuals with severe symptoms of diarrhoea than assemblage B infection (Haque *et al*., 2009; Breathnach *et al*., 2010; Ignatius *et al*., 2012). Other studies reported assemblage B infection in individuals with severe diarrhoea and in asymptomatic carriers (Homan and Mank, 2001; Gelanew *et al*., 2007; Sahagun *et al*., 2007; El Tantawy and Taman, 2014). As such, there are inconsistencies in results being reported by various studies regarding *Giardia* assemblages and their clinical manifestations of symptoms (Sahagun *et al*., 2007; Sprong *et al*., 2009; Feng and Xiao, 2011; Choy *et al*., 2014; Thompson and Ash, 2016).

2.9 Diagnosis

Globally, *Giardia* parasites can be diagnosed by using different methods which include microscopy, antigen – antibody tests and molecular assays (Tashima *et al*., 2009; Mark-Carew *et al*., 2010; Abe and Teramoto, 2011; Hawash, 2014).

2.9.1 Microscopic methods

Globally, microscopy is one of the traditional ways of diagnosing *Giardia* infection through the visualization of *Giardia* cysts and trophozoites (Hawash, 2014; Elmi *et al*., 2017). It is considered as the 'gold standard' as far as diagnosis of *Giardia* infection is concerned *(*Weitzel *et al*., 2006; den Hartog *et al*., 2013). Microscopy is the mainstay of diagnosis in many health facilities in Africa and Zambia inclusive (Nkrumah *et al*., 2011; Mwanakasale *et al*., 2018). The commonly performed microscopic methods include the Zinc Sulphate floatation and formol ether concentration techniques (Zajac *et al*., 2002). The methods are reliable, but less sensitive when dealing with low *Giardia* cyst count (Feng and Xiao, 2011). However, their advantage lies in detecting other intestinal parasites that may be present in faecal samples (Schuurman *et al*., 2007). The microscopic formol ether concentration technique has a detection rate of 60-80% for single faecal sample analysis, 80-90% and over 90% for analysis of two or three faecal samples (Gardner and Hill, 2001), respectively.

2.9.2 Antigen detection methods

There are several detection methods that target *Giardia* antigens in faecal samples (Johnston *et al*., 2003; Weitzel *et al*., 2006) such as the enzyme-linked immunosorbent assays (ELISAs), immunofluorescence antigen tests (IFA) and rapid antigen detection tests (RDTs) (Heyworth, 2014), respectively. Antigen tests are more sensitive than microscopy (Al-Saeed and Issa, 2010)

and have sensitivity of 95 to 100% and specificity of 100% (Pestechian *et al*., 2014). Thus, a number of health facilities worldwide use antigen based methods to diagnose *Giardia* infection as they are reliable and require much less time to run several tests (Ng *et al*., 2005; Schuurman *et al*., 2007; Christy *et al*., 2012). The results are comparable to those reported by sensitive faecal-based PCR assays (Schuurman *et al*., 2007). However, antigenic techniques cannot be used to differentiate *Giardia* species (O'Handley, 2002; Gaafar, 2011).

2.9.3 Molecular methods

Globally, use of molecular assays has greatly improved the understanding of taxonomy, population genetics and epidemiology of *G. duodenalis* infection (Feng and Xiao, 2011). The molecular assays include the polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) and DNA sequence analysis (Read *et al*., 2004; Cacciò *et al*., 2008; Wang *et al*., 2014; Wang et *al*., 2016). Their sensitivity is very high and capable of detecting as low as 10 *Giardia* cysts in a faecal sample, that is 100% for 4 -10 cysts; 94% for 3 cysts; 90% for 2 cysts; and 80% for 1 cyst (Amar *et al*., 2002; Mcglade *et al*., 2003; Guy *et al*., 2004; Miller and Sterling, 2007; Hawash *et al*., 2016). However, molecular assays are currently being used for research purposes in many laboratories globally (Smith and Mank, 2011; Fantinatti, 2019).

2.9.3.1 Genotyping using genetic markers

Molecular characterization of *G. duodenalis* to assemblage or sub-assemblage level can be achieved by using specific genetic markers (loci) or genes (Sulaiman *et al*., 2003; Abe *et al*., 2005; Wielinger and Thompson, 2007; Ryan and Cacciò, 2013). These genetic markers include the Small subunit ribosomal RNA (ssu-rRNA) (Appelbee *et al*., 2003), βeta-giardin (*bg*) (Cacciò

et al., 2002; Lalle *et al.*, 2005), glutamate dehydrogenase (*gdh*) (Read *et al*., 2004; Cacciò *et al*., 2008) and triose phosphate isomerase (tpi) genes (Bertrand *et al*., 2005; Sprong *et al*., 2009).

2.9.3.2 Genotyping using the *gdh* gene

The *gdh* gene is one of the commonly used genetic markers to genotype *G. duodenalis* isolates from humans and other vertebrates (Read *et al*., 2004; Ryan and Cacciò, 2013; Peng *et al*., 2016; Strkolcova *et al*., 2016). It is able to characterize *G. duodenalis* isolates into assemblages A or B and further splitting them into sub-assemblages AI, AII, BIII and BIV (Amar *et al*., 2002; Read *et al.*, 2004). It is capable of identifying mixed infections associated with either assemblages or sub-assemblages (Homan and Mank, 2001; Read *et al*., 2002). As such, the *gdh* gene is reliable, easy and cost-effective in genotyping *G. duodenalis* isolates directly from faecal samples (Pestehchian *et al*., 2012).

2.10 Treatment of *Giardia* infection

On the global scale, six classes of drugs have been approved for treatment of giardiasis in humans and these include the 5-nitroimidazole (metronidazole [MTZ; Flagyl], tinidazole), nitazoxanide, paromomycin, furazolidone, quinacrine, and benzimidazole (albendazole) derivatives (Lalle, 2010) as shown in Table 2.3. The first-line drugs comprise of metronidazole, tinidazole and nitazoxanide (Nash *et al*., 2001; Lane and Lloyd, 2002; Savioli *et al*., 2006). The second line (alternative) drugs include paromomycin, furazolidone, quinacrine and albendazole (Harris *et al*., 2001; Escobedo and Cimerman, 2007). Post infectious irritable bowel syndrome

can be managed by using drugs like Tegaserod and Prucalopride and also reducing in-take of fiber diet (Spiller, 2005; Salonen *et al*., 2010; Carroll *et al*., 2012; Lyra and Lahtinen, 2012).

Table 2.3: Treatment of giardiasis in humans (Gardner and Hill, 2001; Boarato-David *et al*., 2017)

First line drugs	**Patient type, dosage and duration**
Metronidazole (MTZ; Flagyl)	Adult dosage: 250 mg three times a day for 5 days Pediatric dosage: 15 mg per kilogram of body weight per dose, 3 times per day, for 5 days
Tinidazole (Fasigyn)	Adult dosage: 2 g once Pediatric dosage: 50 mg per kilogram of body weight once (max. 2 g
Nitazoxamide (Alinia or Annita)	Adult dosage: 500 mg two times a day for 3 days Pediatric dosage: 1-3 yrs.: 100 mg every 12 hours for 3 days; 4-11 yrs.: 200 mg every 12 hours for 3 days
Alternative drugs	**Patient type, dosage and duration**
Paromomycin (Humatin)	Adult dosage: 25-35 mg per kilogram of body weight per dose, 3 doses per day for 7 days Pediatric dosage: 25-35 mg per kilogram of body weight per dose, 3 doses per day for 7 days
Furazolidone (Furoxone)	Adult dosage: 100 mg four times a day for 7-10 days Pediatric dosage: 6 mg per kilogram of body weight per dose, 4 doses per day for 7-10 days
Quinacrine	Adult dosage: 100 mg three times a day for 5 days Pediatric dosage: 2 mg per kilogram of body weight, three times per day for 5 days (max. 300 mg/d)
Albendazole (Albenza)	Adult dosage: 400 mg once a day for 5 days Pediatric dosage: 15 mg per kilogram of body weight per day for 5 to 7 days (max. 400 mg)

2.11 Control of *Giardia* infection

Giardiasis can be controlled at individual or community level mainly by practicing good personal hygiene of washing hands thoroughly with soap after using the toilet, before preparing food or

eating and after changing diapers (Sheth and Obrah, 2004; Fewtrell *et al*., 2005; Escobedo *et al*., 2010). At individual or community level, *Giardia* infection can be controlled through proper disposal of human excreta, avoiding contact or contamination with human or animal waste, use of adequately treated/boiled water for drinking and cooking, cooking food thoroughly, washing fruits and vegetables before eating (Ivanov, 2010; Maldonado-Barrera *et al*., 2012). Further, at individual or community level, giardiasis can be controlled through the improvement of housing infrastructure and provision of good toilet facilities (Fantinatti, 2019). Screening of family members and treating individuals who are infected can help stop the spread of *Giardia* infection in endemic communities (Al-Mekhlafi1, 2017). Health education campaigns in schools and communities through the promotion of behavioural change at individual level can help in preventing re-infection and transmission of infection (Inpankaew *et al*., 2007; Brown *et al*., 2013; Mahmud et *al*., 2015; Fantinatti, 2019). Travellers to endemic countries are encouraged to drink bottled water and avoid contact with untreated water bodies (Boarato-David *et al*., 2017).

CHAPTER 3: MATERIALS AND METHODS

3.1 Study design and study site

This was a cross-sectional study conducted between May and September 2017 in selected public and community schools in Chawama, Lusaka District, Zambia. A total of 329 asymptomatic school–going children (146 boys and 183 girls) were sampled from two public schools (123) and two community schools (206) in Chawama. The children's age ranged from 3 to16 years old. The children were categorized into three age-groups, the 3 – 6 years old, 7 – 9 years old and 10 – 16 years old, in line with the average age of children in pre-school, lower primary and upper primary grades, respectively.

Chawama is located on the outskirts of Lusaka City about 3 to 4 Km from the central business district (CBD) area (Figure. 3.1). It lies approximately between latitudes 15^{o} 25'S and 15^{o} 30'S and longitudes 28^{o} 15'E and 28^{o} 20'E and 1200 meters above sea level. Generally, Chawama experiences three seasons; a hot dry season from August to October with temperatures between 26 to 38^{o} C, a rainy season from November to April with temperatures ranging from 27 to 34^{o} C, and a cool dry season from May to August with temperatures from 13 to 20^{o} C. It is one of the densely populated places in Lusaka District with challenges of poor sanitation, erratic and inadequate water supply, vulnerable to *Cholera* outbreaks and other diarrhoeal diseases. In many cases, garbage is left to decompose in undesignated areas along the roads, thereby posing a health hazard to the population. The majority of the population in the area lives in poor standard houses. Chawama has a large population that is heterogeneous with people of different groups and occupational backgrounds. These include civil servants, shop-keepers, marketeers and brick-layers, carpenters and others. The commonly spoken language is Nyanja. The people get their

drinking water from borehole, municipal piped water or through municipal kiosks which have been placed in designated places. Chawama has three public primary schools, several community schools, and one level one - district hospital, one private hospital (specialized in eye diseases) and a police post.

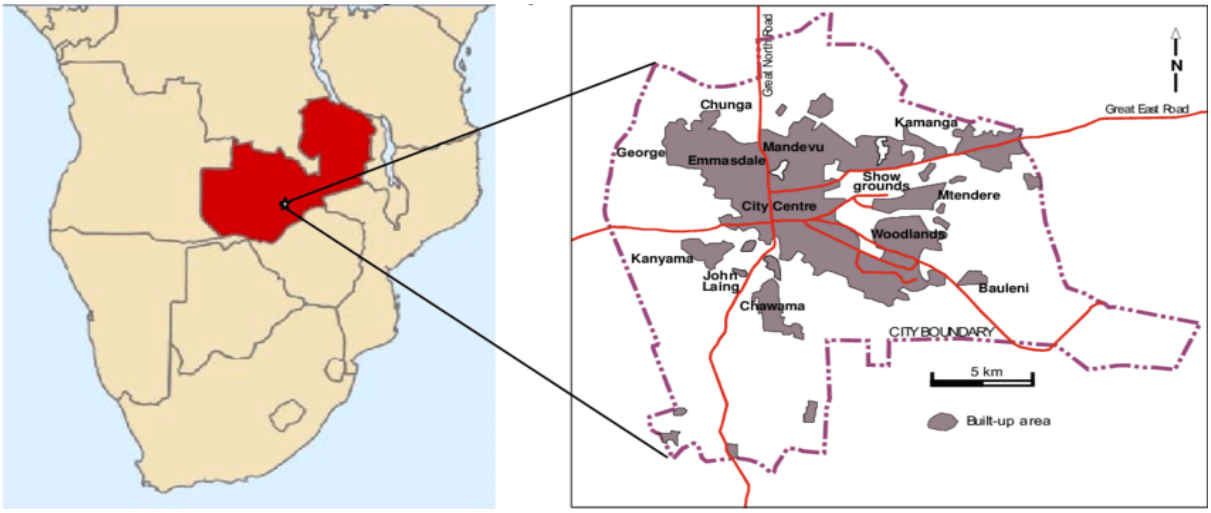

Figure 3.1: Map of Lusaka district showing the study area (Chawama)

3.1.2 Inclusion criteria

All school going-children aged between 3 to 16 years enrolled in selected schools in Chawama were eligible to participate in the study.

3.1.3 Exclusion criteria

Any child who complained of vomiting, diarrhoea, abdominal pain, loss of appetite, weakness, and nausea at recruitment was excluded from the study.

3.2 Sample size determination

The sample size was determined using single population proportion formula described by Aleka *et al*. (2015).

Sample size formula used was as follows;

$n = Z^2 x\ P\ (1-P)/d^2$

Where n= the required sample size,

P= the expected prevalence (0.43) (Roointan *et al*., 2013)

Z= degree of accuracy at 95% (1.96) and

d= margin of error or acceptable accuracy range (0.05).

Calculation

$n= 1.96^2 \text{ X } 0.43(1-0.43)/0.05^2$

n=3.8416 X 0.43 X 0.57/0.0025

n=0.614656/0.0025

n= 376

3.2.1 Sampling method

The study participants and the schools were randomly selected for this study. A total of 800 children were issued with questionnaires prior to being enrolled and at the same time soliciting for consent from their parents/guardians. Out of these 800 children, 400 were from public schools and the other 400 came from community schools. The number 800 of would participants was targeted in order to increase chances of meeting the calculated sample size and taking into consideration of would be drop-outs. Thus, a one month period of sampling and recruitment was allocated for each of the selected schools. Those parents/guardians who satisfied the

questionnaire requirements and gave consent had their children enrolled onto the study. The study sample size was calculated using 43% prevalence rate obtained by Roointan *et al*. (2013) and not the 29% prevalence reported by Siwila *et al*. (2010), this was done in order to achieve high detection rate of *Giardia* infection as cysts are intermittently shed in faeces.

3.2.2 Faecal sample collection

A total of 329 school-going children were enrolled and responded favorably by submitting faecal samples for analysis. One faecal sample was collected in a 50 mL screw-capped container from each study participant following consent from parents/ guardians and upon satisfying the questionnaire requirements. Study participants were instructed to collect faecal samples at their homes by scooping a suitable amount of faecal sample using a provided scoop into the sterile container. The collected faecal sample was delivered on the following day as they came to school by dropping it into a biohazard bag placed in a designated area. After which, the biohazard bag with samples received was properly wrapped and placed into a well secured cooler box for transportation to UTH-Parasitology laboratory. The samples were stored at $4^{o}C$ without preservation until analysis. It took about 4 -5 days from recruitment to giving of a faecal sample by each study participant. It is also important to note that this study was conducted during dry season as it was considered to be the best time for research compared to rain season where accessibility to places become challenging due to flooding.

3.3 Microscopic examination

Stool samples were examined macroscopically for color, consistency, presence of blood and mucous (Cheesbrough, 2005). Sample consistency was reported as formed, semi-formed and

watery (Shiro *et al*., 2017). In Brief, the formal ether concentration method was performed as described by Won *et al*. (2015). Approximately 1 gm of faecal sample was emulsified in 7ml of 10% formal saline. This was followed by filtration of faecal suspension through a gauze layer into a centrifuge tube. After which, approximately 3 ml of diethyl ether was added to the filtrate. The mixture was shaken vigorously for a minute and followed by centrifugation at 750 x g for 3 minutes. Thereafter, the supernatant was discarded, and then a drop of collected sediments was placed on glass- slide under the cover-slip. The stool samples were microscopically examined for *Giardia* cysts under 10 × and 40 × magnification, after staining with Lugol's iodine. *Giardia* infection was graded as described by Almeida *et al*. (2006), 1 – 2 cysts reported as 1+ (very low), 3 – 10 cysts as 2+ (low), 11 – 30 cysts recorded as 3+ (medium or moderate), >30 cysts recorded as 4+ (high).

3.4 DNA extraction

All faecal samples that were *Giardia* positive by microscopic examination were stored at 2 - 8 °C prior to DNA extraction. Extraction of genomic *Giardia* DNA was performed directly from all positive stool samples using Faecal DNA MiniPrep (Zymo Research, California-USA) kit in accordance with the manufacturer's protocol. Briefly, approximately 150mg of faecal sample was pipetted into ZR Bashing bead lysis tubes and 750 µL Lysis solutions were added. The mixture was vortexed for 3 minutes and centrifuged for 1 minute at 10,000 x g. Then 400 µL of supernatant was transferred to Zymo-spin IV spin filter placed in a collection tube and centrifuged at 7000 x g for 1 minute. This was followed by addition of approximately 1,200 µL of DNA binding buffer (with beta-mercaptoethanol) to the filtrate. Then 800 µL of the mixture was transferred to Zymo-spin IIC column placed in sterile collection tube and centrifuged at 10,

000 x g for 1 minute. The flow through was discarded from the collection tube and the above step repeated. Thereafter, 200 µL DNA Pre-wash buffer was added to Zymo-spin IIC column placed in sterile collection tube and centrifuged at 10,000 x g for 1 minute. Then exactly 500 µL of faecal DNA Wash buffer was added to the Zymo-spin IIC Column. Centrifugation was performed for this step as above. Then the Zymo-Spin IIC Column was transferred to a clean 1.5 ml micro centrifuge tube, to which 100 µL of DNA Elution Buffer was added directly to the column matrix and incubated for 5 minutes at room temperature. After incubation, the mixture was centrifuged at 10, 000 x g for 30 seconds. The extracted DNA samples were stored at -20°C until use.

3.4.1 PCR amplification of *gdh* gene

The primers used for PCR amplification are shown in Table 3.1. The amplification of the 432 base pairs (bp) *gdh* gene was carried out in a semi-nested PCR assay using primers and specific conditions as described by Read *et al*. (2004). Briefly, the primary PCR reaction mixtures were modified as below, containing 12.5 µL of One Taq 2 x Master Mix with standard buffer, 1.5 µL of each primer, 7.5 µL of free nuclease water and 2 µL of DNA in a reaction volume 25 µL. The primer pair GDHeF and GDHiR was used in the primary PCR reaction. One microliter of PCR product from the primary PCR reaction was added to the second PCR reaction tube containing primers GDHiF and GDHiR. The modified reaction mixture for second PCR was performed using of 12.5 µL One Taq 2x Master Mix with standard buffer, 1.5 µL of each primer, 8.5 µL of free nuclease water in a total volume of 25 µL.

The DNA was amplified using the Applied Biosystems 2720 Thermal Cycler, under the following PCR conditions, initiated with 2 cycles of 94^{o}C for 2 min, 56^{o}C for 1 min and 72^{o}C for 2 min followed by 45 cycles of 94^{o}C for 30 sec, 56^{o}C for 20 sec and 72^{o}C for 45 sec . Then there was a final extension of 72^{o}C for 7 min, which was followed by storage of PCR products at 4^{o}C. The PCR products were run-on 1.5% agarose gel-electrophoresis stained with ethidium bromide and visualized on ultra violet (UV) transilluminator.

Table 3.1: *Giardia duodenalis gdh* primers and sequences (Read *et al*., 2004)

PCR	Primer	Sequence	Base pair (bp)
First PCR	GDHeF	5' TCA ACG TYA AYC GYC GYT TCC GT3	
	GDHiR	5' GTT RTC CTT GCA CAT CTC C3	-
Second PCR	GDHiF	5' CAG TAC AAC TCY GCT CTC GG3	432
	GDHiR	5' GTT RTC CTT GCA CAT CTC C3	

3.4.2 PCR-RFLP analysis

The RFLP analysis was performed as described by Read *et al*. (2004). Briefly, the NlaIV enzyme (New England Biolabs, USA) reaction mixture contained 10 µL PCR product, 1 µL NlaIV enzyme, 2 µL of 10X enzyme buffer and 17 µL free nuclease water in a final volume of 30 µL. The reaction mixture was incubated for 16 hours at 37°C. The Rsal enzyme (New England Biolabs, USA) reaction mixture comprised of 10 µL PCR product, 1 µL RsaI enzyme, 5 µL of 1X enzyme buffer and 9 µL of free nuclease water in a final volume of 25 µL and incubated for 16 hours at 37°C as well. The NlaIV enzyme was used to distinguish assemblages A, B, C, D and sub-assemblages AI and AII. The RsaI enzyme was used to distinguish sub-assemblages BIII

and BIV of assemblage B. The PCR products and 50 bp molecular marker or ladder (Thermo Fisher Scientific, USA**)** were separated by horizontal electrophoresis using 3% agarose gel stained with ethidium bromide. Visualization of PCR products on electrophoresis gel was done using ultra violet (UV) transilluminator. The predicted fragments (bands) sizes on electrophoresis gel were interpreted according to Roointan *et al*. (2013) as shown in Table 3.2.

Table 3.2: Predicted fragment sizes (bp) and diagnostic genotyping profile of *G. duodenalis* genetic assemblages when digested with Nla IV and *Rsa* I (Read *et al*., 2004).

Assemblage	Enzyme	Predicted fragment sizes	Diagnostic genotyping profile
AI	*NlaIV*(*BspL1)*	16, 18, 39, 87, 123, 149	90, 120, 150
AII	*NlaIV (BspL1)*	16, 18, 39, 72, 77, 87, 123	40, 70, 80, 90, 120
B	*NlaIV* (*BspL1*	18, 123 291	120, 290
BIII	*Rsa1*	2,133, 297	130, 300
BIV	*Rsa1*	I2, 430	430

3.4.3 DNA purification by centrifugation

The PCR products were purified using Promega Wizard® SV Gel and PCR Clean-up system as described by the manufacturers (Promega Corporation, Madison, USA). Briefly, the Quick protocol kit used DNA PCR products obtained after electrophoresis were sliced from gel and placed in a 1.5 mL microcentrifuge tube. Then about 10 uL of Membrane Binding Solution was added per 10 mg of gel sliced. This was followed by vortexing and incubation at 50 – 65^{o}C until gel was completely dissolved. An equal volume of Membrane Binding Solution was added to PCR products. The mixture was transferred to the mini-column assembly, incubated for one

minute and centrifuged at 16,000 x g for one minute. The flow through was discarded from collection tube and minicolumn were replaced into collection tube. This was followed by addition of 700 µL membrane wash solution (10mM potassium acetate pH 5.0, 80% ethanol and 16.7 µM EDTA pH 8.0) to the mini-column and centrifuge at 16,000 x g for a minute. The flow through was discarded and the mini column was re-inserted into the collection tube. The above immediate stage was repeated by addition of 500 µL membrane wash solution and centrifuged at 16,000 x g for five minutes. This was preceded by emptying the column assembly through re-centrifugation for one minute with no lid in order to allow the evaporation of any ethanol residual. The mini column was transferred carefully to a clean 1.5 mL microcentrifuge tube. To which, an addition of 50 µL of nuclease-free water to the mini-column was added and incubated at room temperature for a minute. Thereafter, the purified DNA was centrifuged at 16,000 x g for one minute. The eluted DNA was stored at 4^{o}C or - 20°C until further analysis.

3.4.4 DNA Sequence analysis

Sequencing was performed in both forward and reverse directions using primers GDHiF and GDHiR. All the positive *Giardia* samples were sequenced using the Genetic Analyzer 3130 Applied Biosystems (AB, USA) using the BigDye Kit Standard method. The sequencing mixture contained 0.5 µL Bigdye, 3.75 uL of 5X sequence buffer, 0.33 µL primers, 12.42 µL distilled water and 3 µL DNA template. The cycling conditions were as follows: initial denaturation at 96°C for 1 min, 25 cycles of denaturation at 96°C for 10 seconds, annealing at 50°C for 0.05 seconds and extension at 60°C for 4 minutes and final hold at 4°C.

3.6 Administering of questionnaire

The structured questionnaire was administered to approximately 800 parents/guardians of would be participants. Parents/guardians were asked to answer the questionnaire with closed-ended questions accordingly. The questionnaire contained demographic data on sex, age, source of drinking water, type of toilet facility, contact with pets and type of school. It also contained clinical information on symptoms associated with giardiasis, which included vomiting, diarrhoea, loss of appetite, weakness, epigastric pain and nausea (see attached appendix C).

3.7 Data analysis

3.7.1 Statistical analysis

The demographic data for each child included sex, age, contact with pets, and source of drinking water, type of toilet facility and type of school which were entered into an excel data spread sheet. The entered data were analysed using computer software STATA version 14.0 (StataCorp, College Station, Texas, USA). Prevalence was presented as percentage for all children as well as for the demographic characteristics shown in Table 3.3. The association between dependent variables and independent variables were analysed using Person's Chi-Square test or Fischer's exact test (used when the number of outcomes was less than five). All results were also interpreted using odds ratios, 95% confidence intervals and significance level (P-values < 0.05). Univariate and multivariate Logistic regression was used for analysis of binary outcome (*Giardia* infection or *Giardia* assemblages) and exposure variables (sex, contact with pets, source of drinking water, type of toilet facility and type of school).

Table 3.3: Dependent and independent variables

Dependent variable (Outcome variable)	**Definition of variable**	**Scale of measure**
Having *Giardia* or no *Giardia*	Yes or No	Categorical (binary)
Genotypes (assemblages) or no genotypes	Presence of either assemblage A or B	Categorical (binary)
Independent variable (Predictor variable	**Definition of variables**	**Scale of measure**
Sex	Male/ Female	Categorical (binary)
Age-group (years)	Age attending school	Categorical (Ordinal)
Water source	Municipal tap water, Borehole	Categorical(Nominal)
Contact with pets	Yes or No	Categorical (binary)
Type of toilet	Pit-latrine, flush toilet	Categorical (Nominal)
Type of school	Public, Community	Categorical (Nominal)

3.7.2 Phylogenetic analysis

Phylogenetic analysis was performed in MEGA V6.06 software using the Maximum Likelihood method and the Tamura-Nei evolutionary model (Tamura and Nei, 1993; Tamura *et al*., 2013). Phylogenetic trees were constructed for the *gdh* gene with additional isolates from GenBank. Phylogenetic tree topological reliability was determined using the bootstrap method, with 1000 replicates. The sequences were all aligned manually using ATGC software version 7.5.1 (GENETYX Corporation, Tokyo, Japan). The BLAST program was used for comparing and

analyzing the nucleotide sequences (http://www.ncbi.nlm.nih.gov/BLAST). The bootstrap confidence level used for the phylogenetic trees was 95%. The sequences obtained were then deposited in the DDBJ/GenBank public database under accession numbers LC430549–LC430577 (Table 4.6).

3.8 Ethical considerations

The study (Reference no.007-06-16) was approved by the University of Zambia Biomedical Research Ethics Committee (UNZABREC), Lusaka, Zambia. Permission was sought from the University Teaching Hospital (UTH) Management, UTH - Pathology and Microbiology department, University of Zambia - School of Veterinary Medicine, Ministry of General Education - Lusaka District Education Board Secretary and Head teachers. Prior to commencement of study, the principal investigator held meetings with Head teachers of the respective schools, parents/guardians and school-going children. The purpose, procedures, potential risks and benefits of the study were explained to parents/guardians and school-going children in a language (Nyanja) that is commonly spoken by the people living in Chawama. Parents/guardians were informed that their children could voluntarily withdraw from the study at any time without facing any problems. Written or informed consents were obtained from parents/guardians on behalf of the children prior to enrolment in this study. Human samples inform of faeces were collected for analysis from recruited study participants and processed according to the protocol already outlined above. A unique identification number (ID. No) was assigned to each study participant and on specimen containers. After analysis, children found infected with any intestinal parasitic infection were attended to and treated by assigned medical personnel from Chawama level one hospital. For the sake of confidentiality, privacy and data

protection, the information obtained in this study was only accessible to the principal investigator (PI), research supervisors and assigned medical personnel, respectively.

CHAPTER 4: RESULTS

4.1 Demographic characteristics of study participants

Of the 329 school-going children enrolled, 146 (44%) were males and 183 (56%) were females. The median age of study participants was 10 years, being aged between 3 – 16 years old. The majority of study participants were aged 10 – 16 years old (188/329, 57.1%). Approximately, 123 (37.4%) study participants came from public schools and 206 (62.6%) from community schools as shown in Table 4.1.

Table 4.1: Demographic characteristics of the children

Characteristics	Number of samples (%)
Sex	
Male	146 (44)
Female	183 (56)
Age-category (years)	
3 – 6	36 (10.9)
7 – 9	105 (31.9)
10 - 16	188 (57.1)
Water source	
Municipal tap water	265 (80.5)
Borehole water	64 (19.5)
Type of toilet	
Pit-latrine	301 (91.5)
Water closets	28 (8.5)
Contact with pets	
No	296 (90)
Yes	33 (10)
Type of school	
Public	123 (37.4)
Community	206 (62.6)

4.2 Prevalence of *Giardia* infection and associated demographic factors

The association between the outcome variable (*Giardia* infection) and independent variables (sex, age-category, water source, type of toilet, contact with pets and type of school) were measured by either the chi-square (x^2) or Fisher's exact test as presented in Table 4.2. The

overall prevalence rate of *Giardia* infection was 10.0% (33/329; 95% CI= [0.07 – 0.14]), with prevalence amongst girls being 12% (22/183; 95% CI= [0.50 – 0.61]) and that amongst boys being 7.5% (11/146; 95% CI= [0.39 – 0.49]). By age, the prevalence among the 3 – 6 year olds, 7 – 9 year olds and 10 – 16 year olds was 5.6% (2/36), 9.5% (10/105) and 11.2% (21/188), respectively. *Giardia* infection was more observed in children attending public schools (15.4%, 19/123) than in community school-going children (6.8%, 14/206). *Giardia* infection was significantly ($P = 0.011$) associated with type of school. No statistical significance ($P > 0.05$) was found between *Giardia* infection and other variables sex, age, and water source, type of toilet or contact with pets.

Table 4.2: Prevalence of *Giardia* infection and associated demographic factors

Characteristics	Microscopic results		95% CI	P - value
	Yes (%) (n = 33)	**No** (%) (n = 296)		
Sex*				
Male	11 (7.5)	135 (92.5)	0.39 – 0.49	
Female	22 (12)	161 (87.9)	0.50 – 0.61	0.178
Age-category **(years)				
3 – 6	2 (5.6)	34 (94.4)	0.08 – 0.15	
7 – 9	10 (9.5)	95 (90.5)	0.27 – 0.37	0.690
10 - 16	21 (11.2)	167 (88.8)	0.51 – 0.62	
Water source**				
Municipal tap water	29 (10.9)	236 (89.1)	0.76 – 0.84	0.355
Borehole water	4 (6.3)	60 (93.8)	0.16 – 0.24	
Type of toilet*				
Pit-latrine	28 (9.3)	273 (90.7)	0.88 – 0.94	
Water closets	5 (17.9)	23 (82.1)	0.06 – 0.12	0.149
Contact with pets**				
No	30 (10.1)	266 (89.9)	0.86 – 0.93	
Yes	3 (9.1)	30 (90.9)	0.07 – 0.14	1.000
Type of school*				
Public	19 (15.5)	104 (84.6)	0.32 – 0.43	
Community	14 (6.8)	192 (93.2)	0.57 – 0.68	**0.011**

*****Chi square test was used; ****** Fisher's exact test; n = number; CI = Confidence Interval

4.3 DNA amplification of the *gdh* gene

The 432 bp fragment of the *gdh* gene was successfully amplified in all the 33 DNA positive *G. duodenalis* samples by PCR assay (Figure 4.1). Among these, 22 of the amplified samples were from females and 11 from males, respectively.

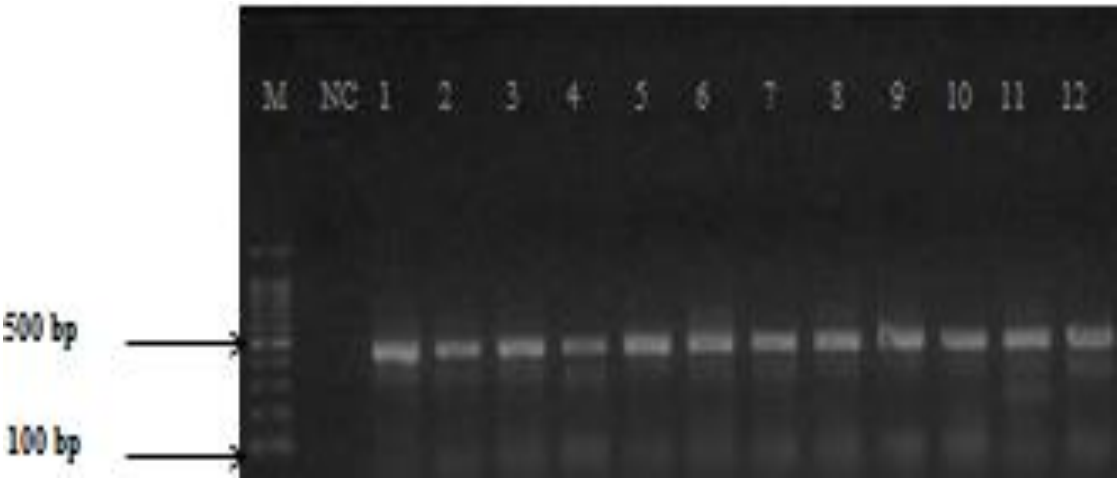

Figure 4.1: Amplification of 432 bp *gdh* genes of *G. duodenalis* isolates. M – 100 bp ladder, NC - Negative control, Lane 1 – 12 – tested samples of *G. duodenalis* on 1.5% ethidium bromide-stained agarose gel electrophoresis at DNA band of 432 bp. The expected fragment size of *G. duodenalis* was 432 as shown in the agarose gel electrophoresis image.

4.4 Genotyping of *Giardia* isolates by PCR-RFLP analysis

Genotyping of *G. duodenalis* isolates was achieved by using the PCR and RFLP analysis, respectively. Genotyping by PCR-RFLP analysis of the *gdh* gene revealed assemblage A (27.3%, 9/33) and assemblage B (72.7%, 24/33) as indicated in Table 4.3. Further, PCR- RFLP analysis showed the division of sub-assemblage AII (27.3%, 9/33), BIII (12.1%, 4/33), BIV (51.5%, 17/33) and mixed BIII/BIV (9.1%, 3/33) infection as shown also in Table 4.3. The digestion of the 432 bp fragment of *gdh* gene by restriction enzyme NIaIV revealed sub-assemblage AII of assemblage A at DNA bands of 90 and 120 bp. The NIaIV enzyme also identified assemblage B at 120 and 290 bp of DNA bands, respectively as shown in Figure 4.2. Further, digestion of

assemblage B with the Rsal enzyme showed sub-assemblage BIII defined with DNA bands at 130 and 300 bp and BIV defined with a 430 bp DNA band as shown in Figure 4.3.

Table 4.3: Genotyping results of *G. duodenalis* by PCR-RFLP analysis

Characteristics	Number	Percentage (%)
Assemblage		
A	9	27.3
B	24	72.7
Sub-assemblage		
AII	9	27.3
BIII	4	12.1
BIV	17	51.5
BIII/BIV	3	9.1

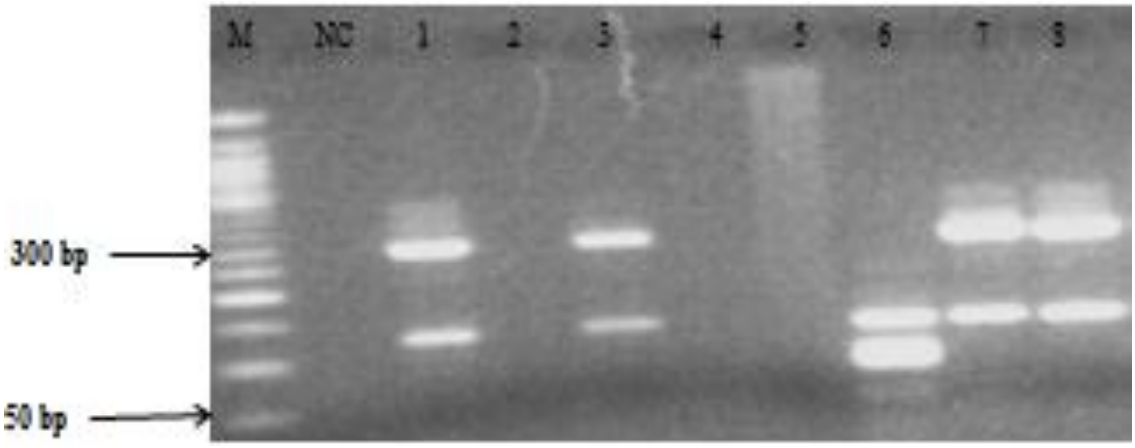

Figure 4.2: NIaIV (Bspl1) digestion of *gdh* –PCR products on 3% agarose gel electrophoresis stained with ethidium bromides. M1– 50 bp Ladder; NC - Negative control , lane 1, 3,7 & 8 - assemblage B at 120 and 290 bp; Lane 6 - sub-assemblage AII at 80, 90 and120 bp, Lane 2, 4 & 5 – negative samples. `

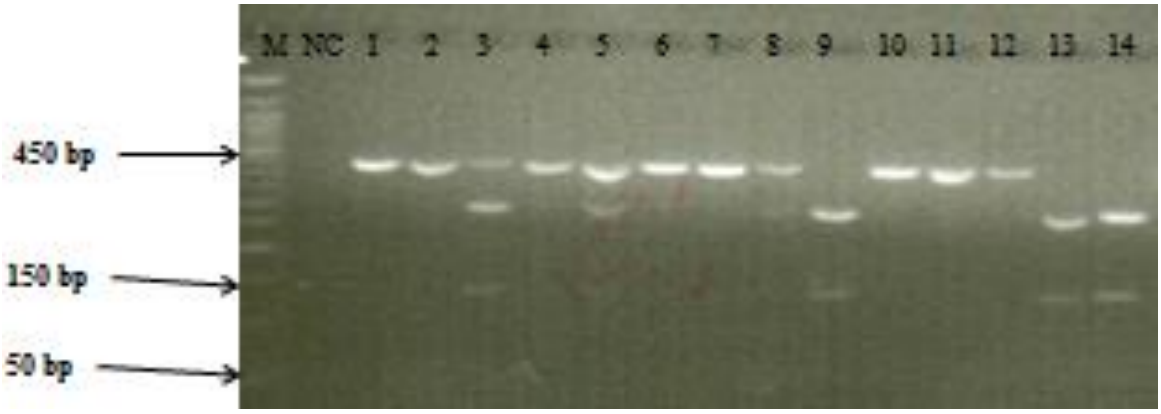

Figure 4.3: Rsal digestion of *gdh* –PCR products on 3% agarose gel stained with ethidium bromide. M – 50 bp Molecular Marker , NC - Negative Control , Lane 1, 2, 4, 6, 7, 10, 11 & 12 - Sub-assemblage BIV at DNA band of 430 bp, Lane 9, 13 & 14 - Sub-assemblage BIII at130bp and 300 bp and Lane 3, 5 & 8 – mixed B at DNA bands of 103, 300 & 430 bp.

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4.5 Prevalence of *G. duodenalis* assemblages

By sex, high prevalence of assemblage B infection was observed in males (10/11, 90.9%; 95% CI= [0.19 – 0.52]) compared to females (14/22, 63.6%; 95% CI= [0.48 – 0.81]). Assemblage B infection was more observed in children aged 7 – 9 year old (8/10, 80 %; 95% CI= [0.17 – 0.49]) compared to other age-groups of 10 – 16 years (15/21, 71.4%; 95% CI= [0.45 – 0.79]) and 3 – 6years (1/2, 50%; 95% CI= [0.01 – 0.23]). No statistical significance (P > 0.05) was found between *Giardia* assemblages and the variables sex or age-groups as shown in Table 4.4.

Table 4.4: Prevalence of *G. duodenalis* assemblages and associated factors

Characteristics	Assemblages		95% CI	P – value
	A n (%)	**B** n (%)		
Sex				
Male	1 (9.1%)	10 (90.9%)	0.18 – 0.52	
Female	8 (36.4%)	14 (63.6%)	0.48 – 0.81	0.212
Age-groups (years)				
3 – 6	1 (50%)	1 (50%)	0.01 – 0.23	
7 – 9	2 (20%)	8 (80%)	0.17 – 0.49	0.695
10 - 16	6 (28.5%)	15 (71.4%)	0.45 – 0.79	

n = number; CI = Confidence Interval

4.6 Phylogenetic analysis of *gdh* gene

From the 33 DNA samples, 29 (87.9 %) isolates were successfully sequenced in order to confirm genotyping results by RFLP (Figure 4.4). The sequences generated in this study were deposited in DDBJ public database with accession numbers LC430549 – LC430577 as assigned in Table 4.6. At the nucleotide level, the *gdh* gene sequences of *G. duodenalis* isolates detected in Zambia shared 89.8% to 100% similarity while the predicted amino acid sequences showed 97.2% to 100% sequence identity. By the Basic Local Alignment Search Tool (BLAST) https://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis the nucleotide sequences of the *gdh* gene of *G. duodenalis* of Zambian assemblage A (as determined by RFLP analysis) strains were highly similar (99 – 100%) to isolate Ad-2 (accession no. L40510) isolated from humans in Australia (Monis *et al*., 1996). Also, Zambian assemblage B *gdh* sequences were highly similar (99 - 100%) to isolate NLH25 (accession no. AY826193), found in humans in the Netherlands (van der Giessen *et al*., 2006). Phylogenetically, the *gdh* gene sequences of *G. duodenalis* were separated into various assemblages and the analysis revealed that 27.6% (8/29; CI = [0.14 –

0.48]) clustered with assemblage A isolates, while 72.4% (21/29; CI = [0.52 – 0.86]) belonged to assemblage B.

Table 4.6: Genotyping by PCR-RFLP and Phylogenetic analysis of *gdh* gene of *G. duodenalis* detected in school-going children in Chawama (n=29/33)

Isolate name	PCR-RFLP genotype		Phylogenetic clustering (Assemblage)	GenBank accession no.
	Assemblage	Sub-assemblage		
ZAM1	B	BIV	B	LC430549
ZAM2	B	BIV	B	LC430550
ZAM3	B	BIV	B	LC430551
ZAM4	A	AII	A	LC430552
ZAM5	B	BIII	B	LC430553
ZAM6	B	BIV	B	LC430554
ZAM7	B	BIV	B	LC430555
ZAM8	A	AII	A	LC430556
ZAM9	B	BIII/BIV	NS	NS
ZAM10	B	BIV	B	LC430557
ZAM11	B	BIV	B	LC430558
ZAM12	B	BIV	B	LC430559
ZAM13	A	AII	NS	NS
ZAM14	B	BIII	B	LC430560
ZAM15	B	BIV	B	LC430561
ZAM16	B	BIV	NS	NS
ZAM17	A	AII	A	LC430562
ZAM18	B	BIV	B	LC430563
ZAM19	A	AII	A	LC430564
ZAM20	B	BIII/BIV	B	LC430565
ZAM21	B	BIV	B	LC430566
ZAM22	B	BIII/BIV	B	LC430567
ZAM23	A	AII	A	LC430568
ZAM24	B	BIV	B	LC430569
ZAM25	B	BIV	NS	NS
ZAM26	A	AII	A	LC430570
ZAM27	B	BIV	B	LC430571
ZAM28	B	BIV	B	LC430572
ZAM29	A	AII	A	LC430573
ZAM30	A	AII	A	LC430574
ZAM31	B	BIII	B	LC430575
ZAM32	B	BIV	B	LC430576
ZAM33	B	BIII	B	LC430577

NS - Not sequenced

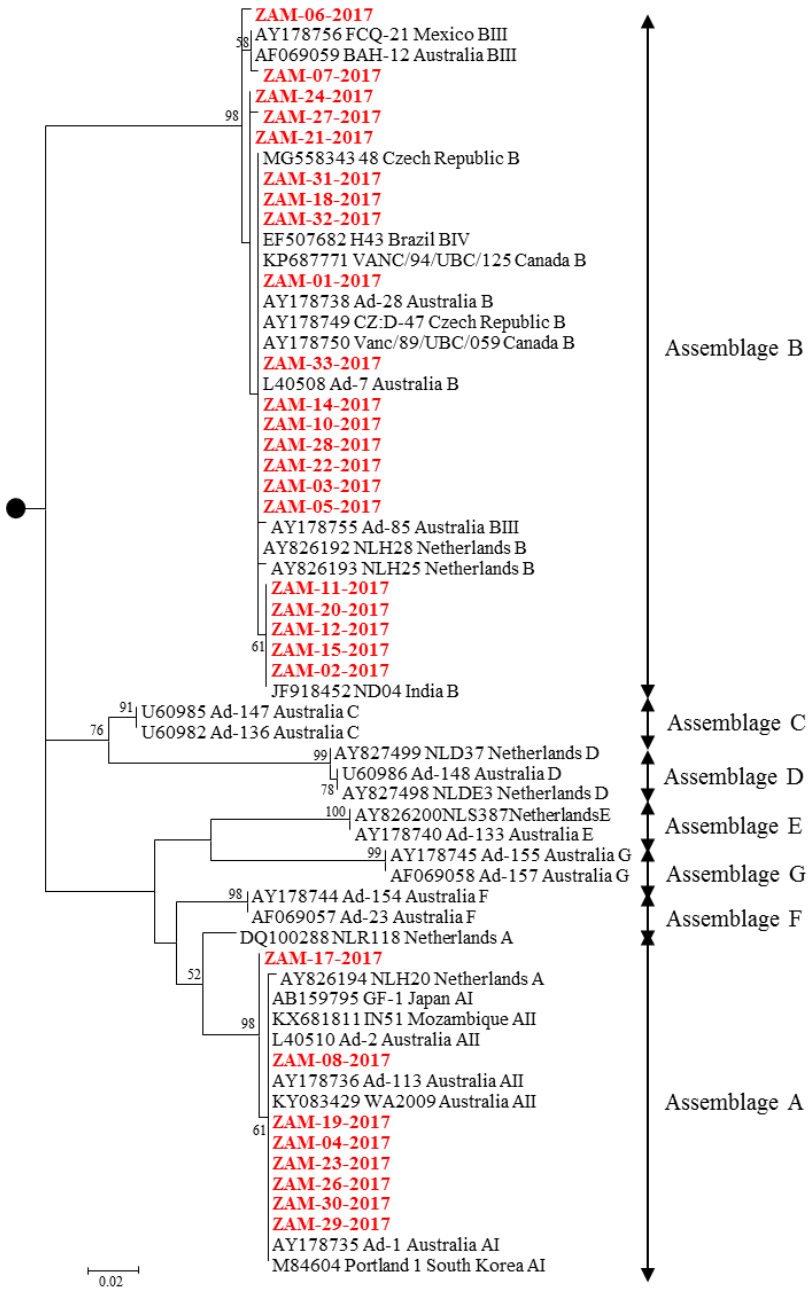

Figure 4.4: Phylogenetic analysis of *G. duodenalis* detected from human samples in Zambia. The analysis was based on the *gdh* gene and involved 63 nucleotide sequences, with a total of 318 positions in the final dataset. The phylogenetic tree was rooted (black circle) to *G. ardeae* (GenBank accession number AF069060). The numbers at branch nodes represent bootstrap values ≥50%. Reference sequences included in the analysis are shown with their respective

GenBank accession numbers, strain name, country of origin and genotype. Parasitic protozoa characterized in this study are shown in bold red text. Bar number of substitutions per site.

4.7 Univariable analysis of risk factors associated with giardiasis

The univariable analysis of risk factors associated with *G. duodenalis* infection is shown in Table 4.5. The variables analysed included sex, age-category, and source of drinking water, contact with pets, type of toilet and type of school. There was no significant association found between *G. duodenalis* infection and sex, age-group, water source, contact with pets or toilet type. The univariable analysis showed that *Giardia* infection was significantly associated with type of school ($p = 0.014$). Further, the univariable analysis showed that children attending public schools were 2.51 times more likely of acquiring *G. duodenalis* (95% CI=[1.21 – 5.20]) compared to community schools.

Table 4.5: Univariable analysis of risk factors associated with giardiasis

Characteristics	COR	95% CI	P-value
Sex			
Male	Ref.	-	-
Female	1.68	0.78 – 3.58	0.182
Age-group (years)			
3 – 6	Ref.	-	-
7 – 9	1.79	0.37 – 8.58	0.467
10 – 16	2.14	0.48 – 9.55	0.320
Water source			
Municipal water	Ref.	-	-
Borehole	0.54	0.18 – 1.60	0.268
Contact with pets			
Yes	Ref.	-	-
No	0.89	0.26 – 3.08	0.850
Type of toilet			
Pit-latrine	Ref.	-	-
Flush toilet	2.12	0.75 – 6.01	0.158
Type of school			
Community	Ref.	-	-
Public	2.51	1.21 – 5.20	0.014

COR = Crude odds ratios; CI = Confidence interval; Ref = Reference

4.8 Multivariable logistic regression analysis of risk factors associated with giardiasis

Multivariable analysis of risk factors associated with *G. duodenalis* infection is shown in Table 4.6. Multivariable analysis still retained only one variable as being significant risk factor of infection among school-going children. After adjusting for sex, age-category, water source, contact with pets and toilet type, multivariable logistic regression analysis showed that the odds ratios of children attending public schools were 2.47 higher of acquiring *G. duodenalis* (95% CI=[1.11 – 5.51]) than those attending community schools

Table 4.6: Multivariable analysis of risk factors associated with giardiasis

Characteristics	AOR	95% CI	P-value
Sex			
Male	Ref.	-	-
Female	1.82	0.84 – 3.96	0.129
Age-group (years)			
3 – 6	Ref.	-	-
7 – 9	1.24	0.24 – 6.39	0.793
10 – 16	1.39	0.29 – 6.75	0.682
Water source			
Municipal water	Ref.	-	-
Borehole	0.44	0.14 – 1.36	0.154
Contact with pets			
Yes	Ref.	-	-
No	1.27	0.34 – 4.83	0.723
Type of toilet			
Pit-latrine	Ref.	-	-
Flush toilet	1.76	0.57 – 5.42	0.324
Type of school			
Community	Ref.	-	-
Public	2.47	1.11 – 5.51	0.026

AOR= Crude odds ratios: CI = Confidence Interval: Ref = Reference

CHAPTER 5: DISCUSSION

This study provides data on prevalence and genotypic characterization of *G.duodenalis* isolates and associated risk factors of infection from asymptomatic school-going children aged 3 – 16 years old in Chawama, Lusaka, Zambia. The microscopic examination of faecal samples showed an overall prevalence of 10% (33/329) of *G. duodenalis* infection. *Giardia* infection was significantly associated with type of school that children attended. Genotyping analysis showed the clustering of *Giardia* isolates into two divisions of assemblages A and B, respectively.

This study reported a low prevalence of *Giardia* infection among school-going children, in agreement with studies done in Thailand (Ratanapo *et al*., 2008; Boontanom *et al*., 2011). This study finding is in contrast with other previous studies done in Zambia and Ethiopia which reported high infection rates of 29 to 55% of giardiasis among school-going children (Graczky *et al*., 2005; Siwila *et al*., 2010; de Lucio *et al*., 2016). The reported low prevalence in this study could be due to several factors: Firstly, it could be attributed to differences in geographical areas studied as this study sampled children from an urban area compared to previous studies which sampled children from a peri-urban and a rural setting (Graczky *et al*., 2005; Siwila *et al.*, 2010), respectively. Secondly, it could be attributed to sampling of asymptomatic children without diarrhoea compared to reporting of high infection rates in children with diarrhoea by previous studies (Graczky *et al*., 2005; Torgerson *et al*., 2015; de Lucio *et al*., 2016). Thus, *Giardia* parasite could be a commensal in asymptomatic individuals living in endemic regions (Bartelt and Platts-Mills, 2016). Thirdly, it could be due diagnostic method used to detect infection (Hussein *et al*., 2017). This study used only microscopic method to detect *Giardia* infection from faecal samples compared to previous studies which used immunofluorescence microscopy and

real-time PCR assay (Graczyk *et al*., 2005; Siwila *et al*., 2010; de Lucio *et al*., 2016). The microscopic method used in this study has a detection rate of 65.26 - 83% compared to immunodiagnostic assays with 100% detection rate (Oguoma and Ekwunife, 2007; Elmi *et al*., 2017; Nooshadokht *et al*., 2017). Last but not the least; it could be due to use of single faecal samples to detect giardiasis rather than the recommended three faecal samples as *Giardia* cysts are intermittently shed in faeces. Analysis of single faecal sample allows the detection of 60 – 80%, whilst analysis of two faecal samples and three faecal samples allow the detection of 80 – 90% and more than 90% (Gardner and Hill, 2001), respectively.

G. duodenalis is a species complex consisting of eight genetic assemblages (A–H) that infect humans and other mammals (Ryan and Caccio, 2013). In this present study, phylogenetic and PCR-RFLP analysis of the *gdh* gene revealed the division of *Giardia* isolates into two major groupings of assemblages A and B. This finding is in agreement with other studies which have reported human infections to be mainly caused by assemblages A and B (Read *et al*., 2004; Faria *et al*., 2016; Mbae *et al*., 2016; Caccio *et al*., 2017; Naguib *et al*., 2018). Assemblage B infection was predominant in this study, similar to previous reports done in Angola, Egypt, Ethiopia, Morocco and Rwanda (Foronda *et al*., 2008; Ignatius *et al*., 2012; Ryan and Caccio, 2013; El Fatni *et al*., 2014; de Lucio *et al*., 2016; Gasparinho *et al*., 2017). Globally, assemblage B is more common than assemblage A in humans (Xiao and Feng, 2017). Its predominance is attributed to infected individuals shedding more cysts in their faeces than those infected with assemblage A (Kohli *et al*., 2008). Contrary to this study, some previous studies in Turkey, Iraq Saudi Arabia and Syria reported the predominance of assemblage A infection (Al-Mohammed, 2011; Tamer *et al*., 2015; Turki *et al*., 2015; Skhal *et al*., 2017). As such, the distribution of

assemblages A and B varies from one country to another (Souza *et al*., 2007; Volotão *et al*., 2007; Caccio and Ryan, 2008; Chanu *et al*., 2018). Their variation can be attributed to parasite factors, which include the rate of multiplication and expression of different surface antigens or host factors such as immunity, past exposure, nutritional status and presence of normal gut flora (Volotao *et al*., 2007; Feng and Xiao, 2011; Cotton *et al*., 2011). However, there was no statistical significance ($P > 0.05$) found between *Giardia* assemblage and variables sex, age-group, water source, type of toilet, contact with pets or type of school in this study, which is in agreement with previous studies (Jerez Puebla *et al*., 2017; Rostaminia *et al*., 2017).

The characterization of *G. duodenalis* isolates by the RFLP analysis of the *gdh* gene further showed the presence of sub-assemblages AII, BIII and BIV infection in this study. This is in agreement with other previous studies (Monis *et al*., 2003; Caccio & Ryan, 2008). The study observed high predominance of sub-assemblage BIV infection, similar to other studies done in Ethiopia, Kenya and Tanzania (Di Cristanziano *et al*., 2014; de Lucio *et al*., 2016; Mbae *et al*., 2016). The detection of sub-assemblages AII, BIII and BIV could suggest anthroponotic transmission in this study. This knowledge of assemblages/sub-assemblages circulating and infecting humans is very important when tracing a source of infection, determining whether it is a re-infection or a new infection (Plutzer *et al*., 2010; Feng and Xiao, 2011; Ferreira *et al*., 2013; Ryan and Caccio, 2013). In addition, understanding the assemblages/sub-assemblages circulating or infecting humans can assist in the implementation of evidence based control measures aimed at preventing human-human transmission by practicing good personal hygiene of washing hands thoroughly with soap after using the toilet or before eating food. However, finding of high sub-assemblage BIV infection in this study was contrary to Sprong *et al*. (2009)

which reported that *Giardia* infections in Africa are mainly caused by sub-assemblage BIII infection.

Furthermore, RFLP analysis of the *gdh* gene in this study revealed mixed infections between sub-assemblages, a finding which has been reported in Ethiopia, Kenya, Palestine and Brazil (Gelanew *et al*., 2007; Faria *et al*., 2016; Mbae *et al*., 2016; Hussein *et al*., 2017). This study reported mixed infections between sub-assemblages BIII/BIV only, in contrast to Mbae *et al*. (2016) study in Kenya that reported infections between AII/BIII and BIII/BIV, respectively. Occurrence of mixed infections appears to be more common than previously thought in humans (Geurden *et al*., 2008; Levecke *et al*., 2009). Thus, their occurrence in this study could be attributed to children being exposed to multiple sources of infection or complex circulation of the parasite in the community or presence of genetically different *Giardia* cysts in faeces. However, mixed infections pose a huge challenge in that detected *Giardia* assemblages cannot be associated with presenting symptoms.

In this current study, *Giardia* infection was reported more in children attending public schools (15.5%, 19/123) than in community school-going children (6.8%, 14/206) and infection was significantly associated with the type of school that children were attending. This is in agreement with a study conducted in Nigeria which attributed its finding to pupils being exposed to dirty playing environments and cultivating in contaminated soils (Esiet and Edet, 2017). Another study in Nepal reported high *Giardia* infection rates among public school-going children (Shrestha *et al*., 2019). This was attributed to low socio-economic status of the families in that they could not afford to provide quality care and good personal hygiene to their children.

However, both univariate and multivariate analysis in this study showed that children in public schools were at greater risk of acquiring giardiasis than those attending community schools. This study observation could be attributed to high enrolments of pupils in public schools compared to low enrolment levels in community schools. High enrolments of pupils do exert more pressure on available amenities, thereby compromising personal hygiene and sanitary conditions. As such, there is need to build new school infrastructure with adequate amenities which could help to alleviate the current high enrolment levels of pupils being experienced in public schools.

This study observed more infections in females than in males, but not statistically significant. This is in agreement with other previous studies done in Portugal, Ethiopia and Ghana (Eyasu *et al*., 2010; Julio *et al*., 2012; Anim-Baidoo *et al*., 2016; Damitie *et al*., 2018). On the contrary, Siwila *et al*. (2010) found sex to be significantly associated with giardiasis; an observation which could be due to other risk behaviours requiring further investigations. Therefore, high infection rates among females in this study could be attributed to poor personal hygiene practices during their involvement in house chores. In some previous studies, high infection rates in females have been associated with their engagement in indoor activities which include changing diapers or washing baby nappies from infected individuals (Eyasu *et al*., 2010; Huruy *et al*., 2011; Abossie and Seid, 2014; Firdu *et al*., 2014).

This study also observed an increase in prevalence of *Giardia* infection with increase in children's age, 3 – 6 years (5.6%, 2/36), 7 – 10 years (9.8%, 17/174) and 11 – 16 years (11.8%, 14/119) in agreement with some previous studies (Al-Mekhlafi *et al*., 2013; Anim-Baidoo *et al*., 2016). This study finding was not statistically significant ($P > 0.05$). This increase in

infection with increase in age in this study could be attributed to children becoming more exposed to playing in dirty grounds and not adhering to good personal hygiene. Indeed, previous studies have shown that *Giardia* infection is most common among children in endemic regions who practices poor personal hygiene (Julio *et al*., 2012; Wegayehu *et al*., 2013; Tappeh *et al*., 2014; Chanu *et al*., 2018; Damitie *et al*., 2018).

The diagnosis of *Giardia* infection can be made using several techniques which include microscopy, immunofluorescence microscopy, immunodiagnostic and molecular assays (Feng and Xiao, 2011; Smith and Mank, 2011; Hooshyar *et al*., 2017; Nooshadokht *et al*., 2017; Ryan *et al*., 2017). Immunodiagnostic tests play a complementary role in the diagnosis of giardiasis because of their high sensitivity and accuracy (Nooshadokht *et al*., 2017). Molecular assays are also highly sensitive in detecting *Giardia* in faecal samples, but they are currently being used for research purposes in many laboratories worldwide (Feng and Xiao, 2011; Ryan and Caccio, 2013; Hooshyar *et al*., 2017). For the reason being that molecular assays require the availability of specialized equipment, well equipped laboratories and specialized trained staff (Elsafi *et al*., 2013; Laude *et al*., 2016).

In conclusion, this is the first study to report on genotypic characterization of *G. duodenalis* isolates from humans in Zambia. Genotypic analysis revealed the circulation of assemblages A and B in school-going children. Sub-assemblages AII, BIII and BIV were detected, suggesting anthroponotic transmission. The overall prevalence of *Giardia* infection was lower than what previous studies reported in Zambia. *Giardia* infection was significantly associated with type of school and that children attending public schools were at greater risk of acquiring giardiasis than

those in community schools. Therefore, control measures aimed at improving sanitary environments in schools or implementation of health education campaigns and introduction of screening programmes would help reduce the spread of human-human infections.

5.1 Recommendations

1. The use immunofluorescence microscopy in routine diagnosis of *Giardia* infection is recommended because of its high sensitivity in detecting infections.

2. The examination of three faecal samples in routine diagnosis of *Giardia* infection is also recommended because of its increased detection rate of infection as *Giardia* cysts are intermittently shed in faeces of individuals suspected to be infected.

3. The Ministry of Health in collaboration with the Ministry of General Education to implement health education campaigns in schools so that pupils understand the modes of transmission of intestinal parasitic infections and how to prevent re-infection.

4. The Ministry of Health in collaboration with the Ministry of General Education to introduce screening programmes on intestinal parasitic infections in schools so that asymptomatic carriers are detected to be treated who might pose a risk to other health individuals.

5.2 Study limitations

1. The use of ordinary microscopy to detect *Giardia* infection, instead of immunofluorescence microscopy may be considered a limitation in this study. The sensitivity for ordinary microscopy is lower than that of immunofluorescence microscopy.

2. The use of single faecal sample rather than the recommended three faecal samples to detect *Giardia* infection may have contributed to the reported low prevalence of infection in this study.

As single faecal sample analysis allows low detection of infection compared to analysis of three faecal samples.

3. The lower sample size collected may have an effect on the obtained results in this study, instead of the calculated sample size due to a limitation of time to recruit study participants as schools during term three of the educational calendar had started preparing for the end of year final grade seven examinations.

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APPENDICES

APPENDIX A: INFORMATION SHEET

Prevalence and genotypic characterization of G*iardia duodenalis i*solates and associated risk factors from asymptomatic school-going children in Chawama, Lusaka, Zambia

Introduction

You are humbly being asked to participate or have your child/dependant participate in the study mentioned above. I would like you to understand the benefits, risks and what is expected of you before you decide to take part in the study. The information sheet gives information about the study procedure. Once you have understood the study and you agree to participate, you will be requested to sign or put your thumb print where indicated in the presence of a witness. Participation is entirely voluntary and you may refuse to have your child /dependant participate.

Purpose of study

Globally, this parasite is one of the most common causes of diarrhoea in humans with an estimation of about 280 million cases per year being reported. Approximately 200 million people are infected with this parasite in Asia, Africa and Latin America with about 500,000 new cases being reported each year. However, the infection caused in some individuals does not present with any clinical symptoms or signs.

The infection transmission is through ingestion of the parasite in contaminated food or water and also through direct faecal-oral route.

The purpose of this study is to determine the impact of infection and to know the genotypes causing infection in children.

Study procedure

The research project was submitted to the University of Zambia Biomedical Research Ethics Committee for approval. Permission was sought from District Education Board Secretary (DEBS) in Lusaka District.

Permission was also obtained from the UTH management and School of Veterinary Medicine for the analysis of sample at the University of Zambia.

All participants recruited in this study were to submit one stool sample in the container that was provided.

Possible benefits

Treatment of study participants found positive for giardiasis will be provided accordingly through the assistance from the Lusaka District Health Office.

Knowledge gained will help in coming up with better control strategies by both Ministry of Education and Ministry of General Education.

Risk/discomfort associated with the study

There will be no risks or discomfort that will be inflicted upon the participants as stool will be collected in a normal way.

Contact person

For any queries concerning the study, please contact Mr. Shadreck Tembo, the Principal Investigator of the study, on mobile number 097625060 or email: s.tembo@unza.zm.

ETHICS COMMITTEE CONTACT ADDRESS

The Chairperson

University of Zambia Biomedical Research Ethics Committee (UNZABREC)

Ridgeway Campus

P. O. Box 50110

Lusaka, Zambia

Telephone: 260-1-256067

Fax: +260-1-250753

E-mail: unzarec@unza.zm

APPENDIX B: CONSENT FORM

Parent/guardian consent

I have been fully informed and explained to about this study and its benefits, I therefore allow my child to participate in the study.

Please sign your name or put your thumb print

Name of the child: ……..

Date: ……

Witness name: ……

Witness signature or thumb print: …….

Date: …….

7.3 Assent form

Children assent (for those who are eight years and above)

I have been fully informed and explained to about this study and its benefits; I agree to participate voluntarily in the study.

Please sign your name or put your thumb print

Name of the child: ……..

Thumbprint: ……

Witness name: ……

Witness signature or thumb print: …….

Date: …….

APPENDIX C

QUESTIONNAIRE AND PERMISSION LETTERS

Study title: Prevalence and Genotypic Characterization of G*iardia duodenalis* Isolates and associated risk factors from asymptomatic School-Going Children in Chawama, Lusaka, Zambia

Province: ……

District: ……..

Name of School: ……

Instructions

You are kindly asked to answer all the questions in this questionnaire.

Participant's Details:

1. Name of the child: …….
2. Sex: Male/Female: …… **....**
3. Age: ……
4. Area of residence: ……..
5. House No: ……..
6. Level of child's education (Nursery/Primary)
7. Level of parent's/guardian's education (Primary/Secondary/Tertially)
8. Do your parents/guardians work? Yes/No

   If yes, what type of employment? Formal employment/ informal employment

9. What is the source of drinking water Municipal tap water/Borehole?
10. Do you keep pets or animals? Yes/No

    If yes, what type of pets or animals? …….

11. Type of toilet Pit-latrine/flush toilet

Child's clinical information:

To a child or parent/guardian

In the past one month did you or your child, experience any of the following symptoms?

a) Vomiting	Yes/No
b) Diarrhoea	Yes/No
c) Abdominal pain	Yes/No
d) Loss of appetite	Yes/No
e) Weakness	Yes/No
g) Epigastric pain and	Yes/No
h) Nausea	Yes/No

THE UNIVERSITY OF ZAMBIA

BIOMEDICAL RESEARCH ETHICS COMMITTEE

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Ridgeway Campus
P.O. Box 50110
Lusaka, Zambia

Assurance No. FWA00000338
IRB00001131 of IORG0000774

24th August, 2016.

Our Ref: 007-06-16.

Mr. Shadreck J. Tembo,
University of Zambia,
School of Medicine,
Department of Biomedical Sciences,
P.O Box 50110,
Lusaka.

Dear Mr. Tembo,

RE: RESUBMITTED RESEARCH PROPOSAL: **"GENOTYPIC CHARACTERIZATION OF GIARDIA DUODENALIS ISOLATES FROM CHILDREN BEING ATTENDED TO AT SELECTED HEALTH CENTRES IN LUSAKA DISTRICT" (REF. No. 007-06-16)**

The above-mentioned research proposal was presented to the Biomedical Research Ethics Committee on 17th August, 2016. The proposal is approved.

CONDITIONS:

- This approval is based strictly on your submitted proposal. Should there be need for you to modify or change the study design or methodology, you will need to seek clearance from the Research Ethics Committee.
- If you have need for further clarification please consult this office. Please note that it is mandatory that you submit a detailed progress report of your study to this Committee every six months and a final copy of your report at the end of the study.
- Any serious adverse events must be reported at once to this Committee.
- Please note that when your approval expires you may need to request for renewal. The request should be accompanied by a Progress Report (Progress Report Forms can be obtained from the Secretariat).
- **Ensure that a final copy of the results is submitted to this Committee.**

Yours sincerely,

Dr. S.H Nzala
VICE-CHAIRPERSON

Date of approval: 24th August, 2016. **Date of expiry:** 23rd August, 2017.

All correspondence should be addressed
to the District Education Board Secretary

Telephone: 0211-240250/240249/0955 623749
Email: desbsisk@yahoo.co.uk

LSK/DEB/101/1/19

REPUBLIC OF ZAMBIA

MINISTRY OF GENERAL EDUCATION

DISTRICT EDUCATION BOARD SECRETARY
P. O. BOX 50297
LUSAKA

15th June, 2017

The Headteacher
Primary/Community School
Chawama Zone

Dear Sir/Madam,

RE: PERMISSION TO CONDUCT IN MSC MEDICAL PARASITOLOGY REASEARCH PROJECT

The subject matter refers.

I write to introduce Mr. Shadreck Tembo an MSC Medical Parasitology student at the University of Zambia of Health Science, who has requested to conduct a research.

Permission has been granted to him to undertake a research project in your schools.

Kindly welcome and cooperate with him.

DEBS

K.K. Mwale (Mr.)
DISTRICT EDUCATION BOARD SECRETARY
LUSAKA DISTRICT

/ss