PREVALENCE, RISK FACTORS AND CHARACTERISATION OF *CRYPTOSPORIDIUM* SPECIES IN CHILDREN PRESENTING WITH DIARRHOEA AT URBAN AND RURAL HEALTH CENTRES IN ZAMBIA

By

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A Dissertation Submitted to the University of Zambia in Partial Fulfilment of the Requirements for the Degree of Master of Science in Medical Parasitology

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DECLARATION

I, Barbara Banda, do declare that this dissertation represents my own work and has not been submitted elsewhere for any other degree or qualification.

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CERTIFICATE OF APPROVAL

Dissertation Title: Prevalence, risk factors and characterisation of *Cryptosporidium* Species in children presenting with diarrhoea at urban and rural health centres in Zambia.

This Dissertation of Barbara Banda has been approved in partial fulfilment of the requirements for the degree of Master of Science in Medical Parasitology at the University of Zambia.

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ABSTRACT

Background

Cryptosporidium species are among the leading causes of diarrhoeal disease especially in children and immunocompromised individuals. The pathogen disproportionately affects settings without adequate sanitation and hygiene especially in developing countries and its genotype isolates vary from place to place. Children under the age of five suffer severe consequences when affected. The study aimed to determine prevalence, risk factors and characterise *Cryptosporidium* species in children presenting with diarrhoea at urban and rural health centres in Zambia.

Methods: This was a Cross sectional study in under five children presenting with diarrhoea. Stool samples collected from 490 children aged <5years with diarrhoea were assessed for *Cryptosporidium* oocysts microscopically after concentration with formal ether concentration technique. A single stool sample was collected from each child. A structured questionnaire was used to collect demographics and socioeconomic characteristics. Positive samples were subjected to Polymerase Chain Reaction (PCR) and partial 60kD glycoprotein (*gp60*) sequence analysis.

Results: The overall prevalence was 10.2% (50/490, 95% CI [7.8 -13.2]) and majority of the infections were in urban areas. Multivariable regression analysis was performed to identify the risk factors of *Cryptosporidium* infection. Children who came from households where boiling water was not practised (AOR 0.74, 95% CI [0.35-1.58]; p=0.439) and those from high density residential areas (AOR 10.20, 95% CI [2.28-45.66]; p=0.002) were more likely to have *Cryptosporidium* infection. Genotyping of 16 positive samples (14 from urban and 2 from rural sources) revealed *C. hominis* (14/16) and *C. parvum* (2/16). The *Cryptosporidium hominis* subtypes identified were Ia, Ib and Ie with subtypes families IeA11G3 (1), IbA9G3R2 (2), IaA31R3 (3), IbA9G3 (5), IaA27R3 (1), IaA30R3 (1) and Ia (1). Subtype IbA9G3 and Ia were identified in children from a rural area. *Cryptosporidium parvum* subtypes were IIcA5G3R2 (1) and IIcA5G3a (1).

Conclusions: All the *Cryptosporidium* isolates in this study were *C. hominis* or anthroponotic *C. parvum,* suggesting that currently anthroponotic transmission dominates in Lusaka and the surrounding countryside. Children who came from household where drinking water was not boiled and from high residential areas were more likely to have *Cryptosporidium* infection.

Keywords: Children, *Cryptosporidium*, *gp60* gene, Risk factors, subtypes, Zambia.

DEDICATION

This thesis is dedicated to my children Nambisa Eunice Mwenechanya and Alex Biza Mwenechanya, my mother Mary Kamekela Banda who never stop encouraging me especially in hard times.

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LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
CI	Confidence Interval
COR	Crude odd ratio
DNA	Deoxyribonucleic Acid
GEMS	Global Enteric Multi Centre Study
GP	Glycoprotein gene
IFNγ	Interferon gamma
MZN	Modified Ziehl Neelsen
PCR	Polymerase Chain Reaction
RHC	Rural Health Centre
SPPS	Species
SSU	Small Sub unit
TNFα	Tumour Necrosis Factor alpha
UNZABREC	University of Zambia Biomedical Research Ethics Committee
UTH	University Teaching Hospital
WHO	World Health Organisation

CHAPTER 1: INTRODUCTION

1.1 Background

Cryptosporidiosis is a diarrhoeal disease of human and animals caused by *Cryptosporidium* species (Valenzuela *et al.*, 2014). These parasites belong to the phylum Apicomplexa, are ubiquitous in the environment and are globally distributed (Domenech *et al.*, 2011, Shirley *et al.*, 2012). The Global Enteric Multicentre Study (GEMS), determined that *Cryptosporidium* species are among the many causes of diarrhoea in children, others include *Rotavirus*, *Shigella* and *Escherichia coli* (Kotloff *et al.*, 2013).

Transmission of *Cryptosporidium* occurs through the faecal-oral route, either indirectly by accidental ingestion of contaminated water or food or by direct contact with infected individuals, animals or fomites (Feng and Xiao, 2011, Ryan *et al.*, 2014). In immunocompromised individuals, cryptosporidiosis typically results in transient (up to 2-3 weeks), self-limiting illness characterised by watery diarrhoea, abdominal pain and less frequently, nausea, vomiting, fever and weight loss (Asma *et al.*, 2011, Domenech *et al.*, 2011; Siwila *et al.*, 2011; Kurniawan *et al.*, 2013, Bouzid *et al.*,2013). The genus *Cryptosporidium* species consist of over 45 spp but only a few cause infections in humans including *C. parvum, C. hominis, C. felis, C. ubiquitum, C. muris, C. meleagridis* and *C. canis. Cryptosporidium parvum* and *C. hominis* are responsible for 90% of human infections, especially in resource-poor settings (Xiao *et al.*, 2010, Zahedi *et al.*, 2021). *Cryptosporidium parvum* consist of IIa-IIt subtypes, of which IIb, IIc and IIe have been detected in human and their transmission appears to be anthroponotic, but IIa and IId are transmitted through zoonotic routes. *Cryptosporidium hominis* consist of subtypes Ia-In (Xiao *et al.*, 2012, Widmer *et al.*, 2020).

The disease burden attributed to anthroponotic transmission of *C. parvum* was likely previously underestimated (Soba *et al.*, 2008). There is new evidence suggesting that some subtypes of *C. parvum* are exclusively circulating in human populations (Xiao, 2010). For instance, *C. parvum* subtype IIc has only been isolated from humans in developing countries and was the most common cause of anthroponotic transmission.

Previous work in Zambia using modified Ziehl-Neelsen microscopy has shown a prevalence of 18-26%, associated with risk factors such as inadequate sanitation and

hygiene (Nchito *et al*., 1998), as well as malnutrition (Amadi *et al*.,2001). A more recent study in humans in Zambia identified *C. hominis*, (59%), *C. parvum* (38%), *C. felis* (1%) and *C. meleagridis* (1%) species using PCR (Mulunda *et al*., 2020).

Therefore, it was essential to first determine the prevalence and compare the proportion and distribution of *Cryptosporidium* species/ subtypes in children under five residing in both urban and rural settings.

1.2 Statement of the problem

Cryptosporidiosis is the infectious disease that cause diarrhoea especially in young children and in immunocompromised individuals worldwide (Kotloff *et al*., 2013). Globally, acute *Cryptosporidium* infections are estimated to cause 48,000 annual deaths in children under five years (Khalil *et al*., 2018). The disease is considered the second greatest cause of diarrhoea and death in children in Africa and Asia (Striepen, 2013). Cryptosporidiosis can go beyond the initial acute diarrhoeal episodes due to the effects on the gastrointestinal mucosa causing villous blunting with chronic inflammation leading to environmental enteropathy (Bartelt *et al.,* 2013). *Cryptosporidium* infection can impede the nutrient absorption and thereby cause malnutrition leading to long lasting cycles of reinfection (Checkley *et al*., 2015). Cryptosporidiosis is also associated with prolonged or persistent diarrhoea, subsequent impairment of growth, physical fitness, stunting and cognitive function (Rodriquez *et al*., 2011, Desai *et al*., 2012, Delahoy *et al*., 2018). The genotype/subtypes circulating among Zambian children causing health impacts is unknown.

1.3 Justification of the Study

Molecular studies in Africa have demonstrated the distribution of *Cryptosporidium* species and subtypes infecting humans (Squire and Ryan, 2017). In Zambia, a recent study by Mulunda *et al*., (2020), used PCR to identify *Cryptosporidium* spp and subtypes in human samples but did no compare the species and genotypes in children under five from urban and rural areas of Zambia (Mulunda *et al.,* 2020). Therefore, there is a knowledge gap which necessitated conducting this molecular study in children to know which spp/subtypes are in circulation and are infecting children in urban and rural areas of Zambia. Characterisation of *Cryptosporidium* spp has been reported to be a useful method in understanding the differences in transmission dynamics, the significance of the subtypes/ strains in humans and their clinical

manifestations associated with the disease (Xiao, 2010). Knowing and understanding the spp/subtypes circulating in children is crucial in tracing source of infection. The study also intends to identify the risk factors associated with *Cryptosporidium* in children in urban and rural areas of Zambia. The knowledge gained could potentially provide data on common genotypes/subtype circulating among children and will assist in decision making in relation to prevention of disease from person to person and zoonotic spread.

1.4 Research Questions

1. What is the prevalence of *Cryptosporidium* infection in children presenting with diarrhoea at urban and rural health centres in Zambia?

2. What genotypes of *Cryptosporidium* are circulating in children in urban and rural health centres in Zambia?

3. What are the risk factors associated with *Cryptosporidium* infections in children at urban and rural health centres in Zambia?

1.5 Objectives

1.5.1 General Objective

To determine prevalence, risk factors and characterise *Cryptosporidium* isolates in children presenting with diarrhoea at urban and rural health centres in Zambia.

1.5.2 Specific Objectives

1. To determine the prevalence of *Cryptosporidium* infection in children at urban and rural health centres in Zambia.

2. To characterise *Cryptosporidium* isolates in children at urban and rural health centres in Zambia.

3. To identify the risk factors associated with *Cryptosporidium* infections in children at urban and rural health centres in Zambia.

CHAPTER 2: LITERATURE REVIEW

2.1 Characteristics of *Cryptosporidium* species

Cryptosporidium is a unicellular protozoan parasite in the phylum Apicomplexa (Ryan, 2016). It was discovered by Tyzzer in 1907 who described it as a protozoan parasite (Fayer 2004). *Cryptosporidium* was first recognised as a causative agent of diarrhoea in 1976 (Navin and Juranek 1984). *Cryptosporidium* species attracted attention with large epidemics in industrialised countries while being undiagnosed and neglected in many developing countries (Mor and Tzipori, 2008; Shirley *et al.*, 2012). Based on morphological, biological and phylogenetic differences, 45 *Cryptosporidium* species are currently considered to be valid (Xiao, 2010, Ryan *et al*., 2014, Zahedi *et al*., 2021). Among them, *C. parvum* and *C. hominis* are responsible for more than 90% of human infections worldwide. Less common species include *C. meleagridis*, *C. felis*, *C. canis, C. cuniculus, C. ubiquitum* and C. *viatorum* (Xiao 2010). The disease is usually self- limiting especially in immunocompetent hosts, however it has been shown to induce weight loss, growth stunting, sustained impact on child development and increased mortality rate, even when asymptomatic. (Shirley *et al*., 2012, Kotloff *et al*., 2013). *Cryptosporidium* is well adapted to zoonotic, water-borne and foodborne transmission, by the faecal-oral route (Smith *et al*., 2007).

2.2 Mode of Infection

Cryptosporidium is shed in the faeces of infected persons and animals. People become infected through multiple transmission routes. Infection may be transmitted through person-to-person, ingestion of contaminated water and food, by direct contact with animals and exposure to recreational water in swimming pools (Putignani and Menichella 2010, Feng and Xiao, 2011, Ghenghesh *et al*., 2012, Insulander *et al*., 2013, Ryan *et al*., 2014). Animals become infected by *Cryptosporidium* spp. through eating food or drinking water that is contaminated with *Cryptosporidium* oocysts (Bajer *et al*., 2012). Even if water borne outbreaks have been reported in many countries (Insulander *et al*., 2013), there is evidence that person to person spread is a common mode of transmission especially among children and staff members in nurseries, day care centres and schools (Lee and Greig, 2010).

2.3 Life Cycle of *Cryptosporidium* species

Cryptosporidium infection is initiated by ingestion of oocysts. The mature oocysts undergoes excystation and four motile sporozoites are released (Tripori and Ward 2002). Sporozoites actively attach, invade and become engulfed by host epithelial cells at the luminal surface (Chalmers *et al*., 2011). The sporozoites differentiate and become spherical in shape leading to the production of trophozoites which are type I meront and merozoites. Type I meront contain six or eight merozoites (Bouzid *et al*., 2013). The merozoites invade neighbouring epithelial cells and develop into either trophozoites or Type II meronts which contain four merozoites. Merozoites eventually invade host cells and differentiate into either macrogamete (Ova) or microgamete. After fertilisation of the macrogamete and microgamete, a zygote is formed which differentiate into four sporozoites as oocyst. Two types of oocysts are produced during this cycle, the thin-walled oocysts which are involved in auto-infections and thick walled oocyst which are resistant to many environmental pressures (Buozid *et al*., 2013, Jenkins *et al.,* 2010).

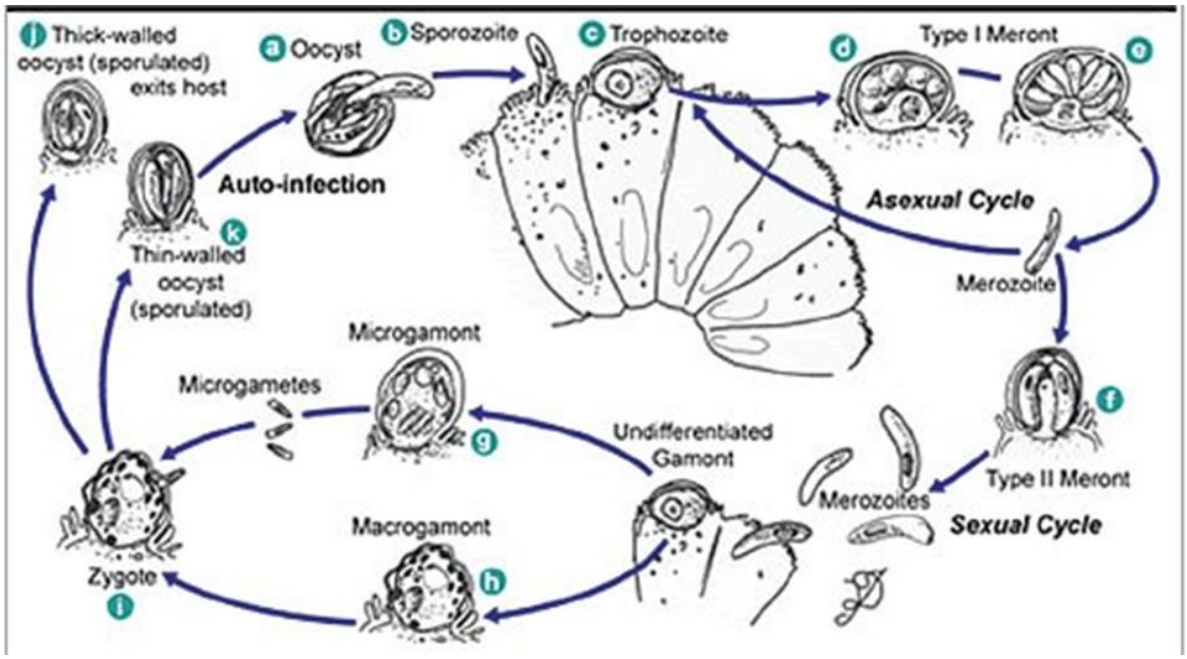

Fig 2.1: Life cycle of *Cryptosporidium.*
(Source: http://www.cdc.gov/parasites/crypto/biology.html)

2.4 Predisposing and risk factors

The predisposing factors of *Cryptosporidium* infection include, lack of appropriate sanitation, lack of toilets, animal contacts/ farm visits, overcrowding, low socio-economic status and poor hygienic conditions, and malnutrition (Mondal *et al*.,2012, Korpe *et al.*,2016, Bouzid *et al*.,2018). Other factors include the intense mobility of persons, high temperatures during rainy seasons (Zheng *et al*., 2018), exposure to human-immunodeficiency virus-infected family members (Parlinac *et al*.,2014), drinking untreated water (Quihui-cota *et al*., 2017), person-person transmission pathway in children attending nursery (Bouzid *et al*., 2018), consuming water during recreation water activities, toileting young children or changing diapers and touching another person with diarrhoea (Caccio *et al*., 2014, Nichols *et al*., 2014). Further, children under five are more likely to be infected with *Cryptosporidium* infection than old ones due to the fact that the vulnerability of younger children and older children are aware interms of insufficient personal hygienic conditions. Besides, younger children have higher chances of being exposed to contaminated sources of infection. In addition, not washing hands before eating and after defecation is a risk of *Cryptosporidium* infection. Exposure to contaminated food and hands is another risk factor (Zheng *et al*., 2018).

2.5 Pathogenesis of *Cryptosporidium* species Infection

After the ingestion of oocysts, sporozoites are released from the excysted oocysts and into the intestinal lumen. The alteration in the intestinal structure and its physiology leads to the pathogenesis of cryptosporidiosis. *Cryptosporidium* infection leads to rapid loss of the microvillus border, shortening and fusion of the villi and lengthening of the crypts which results in malabsorption and diarrhoea due to loss of membrane - bound digestive enzymes, decreased absorption, reduced glucose- sodium chloride absorption and increased chloride anion secretion. Production of proinflammatory cytokines to be specific IFNγ and TNFa also plays a role in the pathogenesis of cryptosporidiosis by increasing production of prostaglandins, neutral peptides and reactive nitrogen intermediates, disruption of the epithelial barrier leading to a leaky and dysfunctional epithelial and alteration of solutes transport leading to osmotic diarrhoea. Prolong diarrhoea and alteration of intestinal barrier function leads to the development of malnutrition in children. (Tripori and Ward 2002; Ajjampur *et al*.,

2008). The severity of the disease depends on the site of inflection, nutritional and immune status of the host and parasites related factors (Chalmers *et al.*, 2010).

2.6 Consequences of Cryptosporidiosis

The consequences of *Cryptosporidium* infection in under five children can extend far beyond diarrhoeal episodes. Subclinical carriage of the parasites especially in developing countries, its consequences are more severe in malnourished than well-nourished children due to the impairment of the T-cell responses (Plattis-Mills *et al*., 2015). Cryptosporidiosis in early childhood is associated with subsequent growth faltering, impaired cognitive development, stunting, physical fitness as well as malnutrition which can be a risk factor to children having recurrent diarrhoeal disease and ultimately death (Ajjampur *et al*., 2010; Mmbaga *et al*., 2017, Squire *et al*., 2017). The association could be mediated by environmental enteropathy or environmental enteric dysfunction, a broad intestinal syndrome which is characterised by local inflammation, nutrient malabsorption, barrier disruption and bacterial translocation due to chronic exposure of the parasites (Opintan *et al*., 2010). Malnourished children are predisposed to infection and they tend to have a higher prevalence of *Cryptosporidium* infection than non-malnourished children. *Cryptosporidium* infection could also impede nutrients absorption thereby causing malnutrition (Checkley *et al*., 2015). Diarrhoea resulting from *Cryptosporidium* infection affects childhood health by decreasing growth of the child (Khalil *et al*., 2018). Several reasons have been advanced that diarrhoea and *Cryptosporidium* could impair physical growth of the child (Rodriquez *et al*., 2011, MAL-ED Network Investigators 2014). The rapid fluid loss and inability to absorb macronutrients and micronutrients during several episodes of diarrhoea disrupts weight and height gain of the child. Furthermore, chronic as well as repeated enteric infections disrupts the normal gut function by changing endothelial cells, causing chronic inflammation and flattening the microvilli which eventually decrease tissue absorption (Weisz *et al.*, 2012, Colombara *et al*., 2016). In addition, failure to meet genetic growth potential could induce poor health outcomes leading to cognitive development, poor education performance and increased risk of cardiovascular metabolic diseases later in life (DeBoer *et al*., 2012, Sudfied *et al*., 2015).

2.7 Diagnosis

Globally, *Cryptosporidium* spp can be diagnosed using different methods, these include microscopy, Immunological assays and molecular methods (Ghaffari and Kalantari 2014, Khurana *et al.,* 2018).

2.7.1 Microscopic examination

2.7.1.1 Modified Ziehl Neelsen

Microscopic detection of oocysts by modified Ziehl Neelsen (ZN) staining in stool samples remains the gold standard for clinical diagnosis of cryptosporidiosis (Savioli *et al*., 2006). This technique uses light microscope and microscopic examination of oocyst is achieved by staining the faecal smear, where the oocysts stain red against blue or green background depending on the counterstain used (Khurana *et al.,* 2018). Unlike other techniques such as ELISA and PCR, ZN technique has the advantages of being the only technique indicating active infections (Abu samra 2013; Omoruyi *et al*., 2014). Modified Ziehl Neelsen is less costly (Abu Samra 2013) and its sensitivity has been estimated at 75% (Chalmers *et al*., 2011).

2.7.2 Immunoassays

There are several methods that target *Cryptosporidium* antigens in faecal samples such as enzyme –linked immunosorbent assay (ELISAs) and immunofluorescent assay (IFA) which have good sensitivity and specificity for the detection of *Cryptosporidium* antigens (Hawash 2014), as well as rapid antigen detection tests (RDTs) (Manouana *et al*.,2020). These immunoassays are widely used either alone or in combination with other techniques mainly for research purposes (Squire and Ryan 2017). Antigen tests are more sensitive than microscopy and have a range of sensitivities and specificities higher than that of microscopic methods (ranging from 58 to 98%) (Hawash 2014). However, these methods cannot distinguish between *Cryptosporidium* species (Elsafi *et al*., 2013)

2.7.3 Molecular methods

The most commonly used molecular method is polymerase chain reaction (PCR), which is more sensitive with the detection range from 1 to 10^6 oocysts (Smith, 2007). The technique involves the amplification of the gene of interest using specific primers that bind to the conserved DNA sequences in *Cryptosporidium* genotypes (Vanni *et*

al., 2012). Various PCR techniques have been developed to identify *Cryptosporidium* spp at molecular level using different markers. These include PCR-restriction fragment length polymorphism (PCR-RFLP), Multiplex allele- specific-PCR (MAS-PCR), Nested PCR and qualitative real time PCR. Molecular assays are currently used for research purposes because they are relatively rapid and have the major advantages of speciation which is important from epidemiological point and in knowing the possible routes of transmission (Khurana and Chaudhary 2018).

2.8 Distribution and Prevalence of *Cryptosporidium* species

Cryptosporidium species in humans differs in distribution between geographical areas and socio- economic conditions worldwide. The difference in *Cryptosporidium* species distribution is mainly due to the routes in which the infection is acquired and the mode of transmission (Xiao, 2010). In a study done in New Zealand, the most commonly species identified were *C. parvum* and *C. hominis*. In Middle Eastern countries, *C. hominis* was the most predominant spp. (Nazemalhosseini-Mojarad *et al*., 2012).

The distribution of *C. parvum* and *C. hominis* between urban and rural areas also differs: *C. parvum* is commonly detected in rural areas whereas *C. hominis* in urban areas (Chalmers *et al.*, 2011, Elbach *et al.*, 2015). Other studies have also observed that there was temporal and age–associated variation in disease burden between *C. parvum* and *C. hominis* species (Chalmers *et al.*, 2011).

Studies have been done on the prevalence of cryptosporidiosis in children and the prevalence varies across countries and between subsets of population (Mor and Tzipori 2008). High prevalence rates of *Cryptosporidium* in children have been reported in countries such as Iraq (43.56%), Turkey (42.8%) (Kuzehkanan *et al*., 2011; Al-alousi 2012). Low prevalence rates were reported in Saudi Arabia (8.3% in-patients and 2.3% outpatients), Kuwait (3.4%), and Philippines (1.9%) (Natividad *et al.*, 2008; Iqbal *et al.*, 2011).

In a recent Zambian study by Mulunda *et al*. (2020) using molecular techniques, the prevalence was observed to be higher in 1-4 years age group than in adults. Studies conducted by Siwila *et al*. in 2010 and 2011 in semi-urban areas, found the prevalence at 28% and 30.7% respectively. Earlier prevalence studies were done by Amadi *et al*. (2001) (prevalence was 26% in children in University Teaching Hospital-UTH with

severe acute malnutrition) and Nchito *et al*. (1998) (prevalence was 18% in school children). These studies were all done using microscopic diagnosis, hence the apparent lower prevalence.

2.9 Genotypes/Subtypes and Clinical Manifestations

Clinical manifestations differ between species and subtypes (Iqbal *et al*., 2011). A study done in Sweden observed that the major clinical manifestations caused by *Cryptosporidium* species were diarrhoea and abdominal pain (Insulander *et al*., 2013). Another study conducted in Barcelona by Segura *et al*. (2015), found that even though all the subtypes were able to cause diarrhoea, the subtype family Ib was the most virulent subtype. According to the English Medical dictionary, virulent is defined as the ability of the pathogen to infect or damage the host immune system. Virulent Subtype IbAI0G2 is associated with both outbreak and sporadic infections.

A study conducted in Kuwait by Iqbal *et al*. (2011) found that *C. hominis* infected children were presenting more clinical manifestation of fever and diarrhoea than children infected with *C. parvum*. However, *C. hominis* subtype Id was associated with severe diarrhoea. A study conducted in Mexico in 12 children found that, altogether seven subtypes belonging to four subtype families of *C. hominis* (Ia, Ib, Id and Ie) and I subtype family of *parvum* (IIa) including IAaI4R3, IaAI5R3, IbAI0G2, IdAI7, IeAIIG3T3, IIaAI5G2RI and IIaAI6GIRI subtypes. (Valenzuela *et al*., 2014). A study conducted in Romania, the two isolated subtypes families were IIa and IId. Subtypes IIdA22GI (n=4) was the single C. *parvum* subtype found in children (Vieira *et al*., 2015). Another study in Spain found four subtypes Id including IbAI0G2 (35), IaA24R3 (6), IIaAI5GIRI (1) and IIaAI5G2RI (Ramo *et al*., 2015).

In another study in Scotland, Deshpande *et al*. (2015), found IbAI02 subtype and four isolates belonging to Ia family (IAaI4R3 (12), IaA9G3 (1), IaA9G3 (1), IaA25R3 (2), two from the Id family (IdA24 (1), IdAI7 (1) and one belonging to the family IeAIIG3T3. Another study conducted in the rural region of western New South Wales, found four subtypes isolated from humans suggesting zoonotic transmission from cattle (Ryan *et al*., 2008).Sow and others (2016) in the Global Enteric Multicentre Study on the burden of *Cryptosporidium* diarrheal disease among children less than 24 Months of age in moderate/high mortality regions of sub-Sahara Africa and South Asia found that anthroponotic IIc and IIe were predominant *C. parvum* subtype families and

of 32 *C. hominis*, the predominant subtypes families were Ia, Ib and Ie and had more diverse subtypes while Ie subtype was exclusively AIIG3T3.

A study conducted in United Kingdom, observed that patients that were presenting with repeated abdominal pain, vomiting and fever were especially infected with the *C. hominis* subtype Ib. Diarrhoea was noted in household members, suggesting person to person transmission (Chalmers *et al*., 2010), though common source exposure is also a possible explanation. A study carried out in rural Nigeria on the molecular characterization of *Cryptosporidium* species, two *Cryptosporidium* species *C. hominis* and *C. parvum* were identified with different subtypes. *C. hominis*-Id (5/6), Ie (1/6) and *C. parvum* IIa (1/2), IId (1/2) were the dominant prevalent species that infected humans (Anejo-okopi *et al.,* 2016). Another study in rural Ghana, (Elbach *et al.,* 2015), subtyping 88 isolates revealed IIcA5G3 (29.6%), IbAI3G3 (19.3%) and IaA2IR3 (13.6%) as the three most frequent subtypes of the spp. Another study in Tanzania, revealed the presence of subtype IfAI2G2 due to anthroponotic transmission (Parson *et al.,* 2014).

In the recent Zambian study by Mulunda *et al*., (2020), *C. hominis* accounted for majority of the cases, with four subtype Ia, Ib, Id, and Ie, and IeII3T3 being the most predominant.

2.10 Treatment and Prevention

Treatment options for cryptosporidiosis are still limited despite evaluation of thousands of drugs (Mead, 2014). Currently, nitazoxanide is the only drug available and it only works well in children who are not infected with HIV (Kelly 2011).

The best strategy to prevent cryptosporidiosis infection is to practice good personal hygiene which includes hand washing before preparing and consuming food, after using a toilet and after coming in contact with diarrhoea patients (Rossle *et al*., 2013), and filtration and clean storage of drinking water (Kelly 2011).

Unfortunately, there is no vaccine for cryptosporidiosis available at the moment.

CHAPTER 3: METHODOLOGY

3.1. Study site/Design

This was a cross sectional study and it was conducted at four locations during August 2018- March 2019. The urban recruitment site was the University Teaching Hospital (UTH) in Lusaka. Chongwe District Hospital (CDH), Ngwerere RHC and Luiimba RHC were chosen to represent rural areas. These study locations/sites were conveniently chosen because the study intended to determine whether there was *Cryptosporidium* spp. variation among children in urban and rural areas and to understand the zoonotic potential to humans.

3.2 Study Population

Children under five years of age presenting with diarrhoea to the selected health institutions were enrolled.

3.2.1 Inclusion Criteria

All children under five years of age presenting with diarrhoea were included in the study.

3.2.2 Exclusion Criteria

Children not presenting with diarrhoea, whose parents or guardians did not provide informed consent and those who were referred to UTH from rural areas were excluded from the study.

3.3 Sample size

The minimum number of samples in this study was calculated using the following sample size formula below (Daniel, 1999):

$n=Z^2P\ (Q-P)/e^2$

Where:

Z= 1.96. The value of Zα required for confidence

p= estimate of the proportion or prevalence of samples containing *Cryptosporidium*

q= Complement of P

e= The precision of the estimate (allowable error), equal half of the confidence interval.

30% was used as an estimate of the proportion or prevalence of samples containing *Cryptosporidium.* 30% prevalence (Siwila *et al.,* 2011)

$n = 1.96^2 \times (0.30 \times (1 - 0.30) / (0.05)^2$

n = 322.7; Thus, 323 stool samples will be examined.

3.4. Sample collection

Paediatric samples were collected from August 2018 –March 2019. Stool samples were collected from UTH, Ngwerere RHC, Chongwe RHC and Luiimba RHC. Convenience sampling was done. This study used this type of sampling to be able to capture the targeted age group of the children in which *Cryptosporidium* spp. infection are known to be common and to have detrimental effects. Leak proof plastic containers were used for sample collection. About 5grams of stool was collected for analysis.

3.5 Laboratory analysis of samples

3.5.1 Formal ether Concentration Technique

Faecal suspension was emulsified in a tube containing 10mls of 10% formal saline. The suspension was mixed well then filtered with 10 mesh screen and poured to conical tubes. About 3mls of ether was added to the tube and strongly shaken, then centrifuged at 3000 rpm for 3 minutes. The supernatant was disposed off and a smear prepared from the sediment (Allen *et al.,* 1970). The smear was then stained with Modified Ziehl Neelsen.

3.5.2 Modified Ziehl Neelsen Staining Technique

Stool samples were emulsified on to a slide by using an orange stick. The smears were dried on the hot plate before fixing them in methanol for 5minutes. After that the smears were placed on the staining rack. The smears were flooded with carbol fuchsin for 10 minutes. The smears were then washed with distilled water and drained. A decolouriser (3% acid-alcohol) was applied on to the smears for 1 minute thereafter, washed with distilled water. The counter stain (1% malachite green or methylene blue) was applied for 1 minute and the smears were then washed with distilled water and placed on the hot plate to dry. Thereafter, immersion oil was mounted onto the slide and examined under the microscope using x 100 objective. *Cryptosporidium* oocysts

appear pink/red against the blue or green background (Henriksen *et al.*, 1981). *Cryptosporidium* positive samples were stored at -20ºC until DNA extraction could be performed for molecular characterisation.

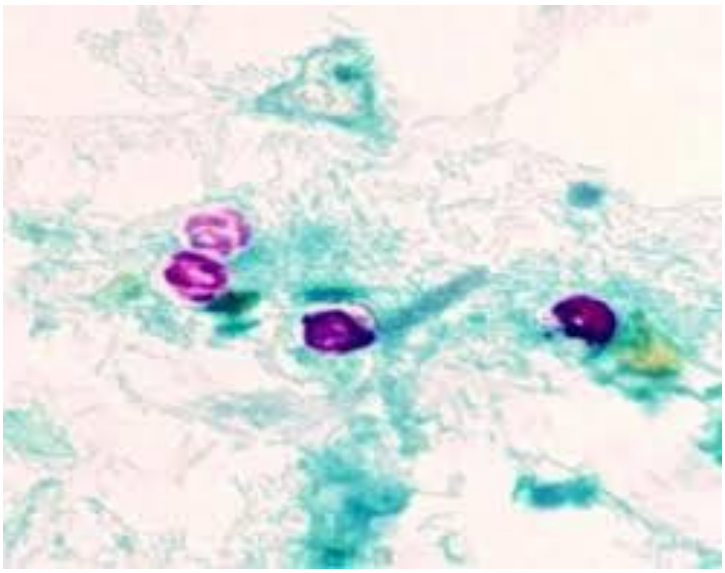

Fig 3:1-Modified Ziehl-Neelsen acid fast staining of *Cryptosporidium* oocysts (Source:https://mcdinternational.org/trainings/malaria/english/dpdx5/html/frames/A-F/Cryptosporidiosis/body Cryptosporidiosis mic1)

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3.6 DNA Extraction

Cryptosporidium genomic DNA was extracted from the faecal sample using a ZR faecal DNA mini kit (Zymo Research, CA, USA), according to manufacturer's instructions. The extracted DNA was quantified using spectrophotometer at wavelength of 260 nm thereafter stored at -20ºC for molecular analysis.

3.6.1 *Cryptosporidium* Genotyping at the *gp60* LOCUS

All the samples which were positive on MZN stain were genotyped by nested PCR technique in which an approximately 850-bp fragment of *gp60* gene was amplified. Primers and amplification conditions used in this study have been described previously (Alves *et al.*, 2003). Positive and negative controls were included in the analysis. Electrophoresis of the PCR products was performed on 1% LE agarose gel containing ethidium bromide. A 100-bp ladder was used as a molecular weight standard and all gels were visualised using Bench top 300 Transilluminator (BioDoc imaging System, CA, USA).

3.6.2 Sequencing Analysis

The PCR products were purified by centrifugation before sequencing using Wizard SV Gel and PCR Clean-up system kit (Promega, Madison, W153711-5399, USA) according to manufacturer's instructions. The products were then analysed on forward and reverse strand using 3500 Genetic Analyser (Applied Biosystem California, USA). The sequences were compared with the published sequences available in the GenBank using the Blast tool and alignment was done using ATGC software. Phylogenetic analysis was done in MEGA7. The neighbour-joining method with the Kimura two-parameter evolutionary model was used to determine the phylogeny (Larkin *et al.,* 2007). All position containing gaps and missing data were eliminated. Sequences from 34 samples which had short sequences were removed and were not included in the phylogenetic tree.

3.7 Administration of Questionnaire

A structured questionnaire was administered to parents and guardians who accepted that their children be part of the study. The questionnaire collected demographic data on sex, age, residence, level of mother and father education, father and mother employment, keeping animals and treatment of water. It also contained questions on clinical symptoms associated with *Cryptosporidium* infection such as if the child had suffered from diarrhoeal disease recently

3.8 Data Analysis

3.8.1 Statistical analysis

All data was analysed using STATA, Version 15.1 (StataCorp, College Station, Texas, USA). For a continuous variable age which was not normally distributed median and interquartile range (IQR) were used. Dependent variable (*Cryptosporidium* infection) was a dichotomous outcome. Therefore, Chi-square test was used in order to understand the association between categorical independent variables with dependable variable if the assumptions were met but where the frequency in any of the cell was less than five (5), fisher's exact test was used. All variables which had p-value of 0.2 or less in binary logistic regression model were collectively entered in the multiple logistic regression model analysis to determine the predictors of *Cryptosporidium* species infection after controlling for possible confounders. For all statistical analysis, a p-value <0.05 was considered statistically significant.

3.8.2 Phylogenetic Analysis

Phylogenetic analysis was conducted in MEGA X (Kumar *et al.*, 2018). The evolutionary history was inferred using the Neighbour- Joining method (Saitou and Nei 1987). The tree was drawn to scale, with branch length in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum composite Likelihood method (Tamura *et al.*, 2004) and are in the unit of the number of base substitutions per site. This analysis involved 24 nucleotide sequences. Codon positions included were 1^{st} +2^{nd} +3^{rd} +Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1077 positions in the final dataset.

3.9 Ethical considerations

Approval to carry out the study was sought from University of Zambia Biomedical Research Ethics Committee (UNZA/BREC), (1^{st} February, 2018, Ref. No.002-11-17). Permission was sought from the UTH Management and Pathology/Microbiology department in order to use their equipment. Permission was also sought from Chongwe District Health Office, Chongwe District Hospital, Ngwerere RHC and Luiimba RHC for collection of stool samples from children (study subjects). The study was done in such a way that privacy and confidentiality was maintained. Confidentiality was adhered to as names of children were not recorded. For purposes of identification, only numbers were assigned for each child (pseudo-anonymisation). After explaining the purpose of the study, informed consent was obtained from parents and guardians. Children with *Cryptosporidium* were treated by the hospital and clinics which they attended.

CHAPTER 4: RESULTS

4.1: Demographic characteristics of the study participants

There were 490 children who participated in this study. The median age of the participants was 18 months (IQR, 12-31). The majority were males (56.5%; 277/490). Most of the study participants were from urban settings (65.9%; 323/490) and majority (230, 47%) were coming from medium density residential areas. More than 60% of the study participants were not boiling their drinking water. Table 4.1.

Table 4.1: Demographic characteristics of study participants

Variable	Category	Proportion (%)	95% CI
Sex	Male	277 (56.5)	52.1 - 60.9
	Female	213 (43.5)	39.1 - 47.9
Health facility	UTH	323 (65.9)	61.7 - 70.1
	Ngwerere	100 (20.4)	16.1 - 24.9
	Luiimba	34 (6.9)	5.0 - 9.5
	Chongwe	33 (6.7)	4.8 - 9.4
Study Location	Urban	323 (65.9)	61.6 - 69.9
	Rural	167 (34.1)	30.0 - 38.4
Residence	Low	73 (14.9)	12.0 - 18.3
	Medium	230 (46.9)	42.5 - 51.4
	High	187 (38.2)	33.9 - 42.5
Father's education	Primary	67 (13.7)	10.9 - 17.0
	Secondary	265 (54.1)	49.6 - 58.5
	Tertiary	158 (32.2)	28.2 - 36.5
Mother's education	Primary	147 (30.1)	26.0 - 34.1
	Secondary	235 (48.1)	43.7 - 52.6
	Tertiary	107 (21.8)	18.5 - 25.8
Father employed	No	192 (39.2)	34.9 - 43.5
	Yes	298 (60.8)	56.4 - 65.1
Mother employed	No	381 (77.8)	73.8 - 81.2
	Yes	109 (22.2)	18.8 - 26.2
Water treatment	Not boiling	296 (60.5)	56.1 - 64.8
	Boiling	193 (39.5)	35.2 - 43.9
Keeping animals	No	359 (73.4)	69.3 - 77.2
	Yes	130 (26.6)	22.8 - 30.7

4.2: Prevalence of *Cryptosporidium* by study location

The overall prevalence rate of *Cryptosporidium* infection was 10.2% (50/490, 95% CI: 7.8-13.2) with the peak in samples that were collected in March which corresponds to the rainy season. Table **4.2** below shows the prevalence of *Cryptosporidium* infection by location.

Table 4.2: Prevalence of *Cryptosporidium* by study location

Variable	Category	*Cryptosporidium*		Total Number (%)	95% CI
		Yes (n=50)	No (n=440)		
Study location	Urban	48 (14.9 %)	275 (85.1 %)	323 (65.9 %)	62.3 - 69.6
	Rural	2 (1.2 %)	165 (98.8 %)	167 (34.1 %)	31.1 - 38.6
	Total	**50**	**440**	**490**	

4.3: Prevalence of *Cryptosporidium* infection and associated demographic variables

Among the total number of children enrolled, the prevalence among males was 11.9% (33/277) and that among females was 7.9% (17/213). *Cryptosporidium* infection was observed more in children coming from urban 14.9% (48/323) than rural 1.2% (2/167) settings. It was found that there was a significant association between *Cryptosporidium* infection with health facilities ($p<0.001$), study location ($p<0.001$), residence ($p<0.001$), water treatment ($p=0.004$) and father's education ($p=0.004$), but there was no association with sex ($p=0.15$) as shown in Table 4.3. Children who were reported to have had experienced a diarrhoea episode were more likely to be infected with *Cryptosporidium* spp. ($p=0.001$).

Table 4.3: Prevalence of *Cryptosporidium* infection and associated demographic factors

Variable	Total Number (%)	*Cryptosporidium*		P-value
		Yes (%) (N=50)	No (%) (N=440)	
Sex				
Male	277 (56.5)	33 (11.9)	244 (88.1)	
Female	213 (43.5)	17 (7.9)	196 (92.0)	0.15
*Health facilities				
UTH	323 (65.9)	48 (14.9)	275 (85.1)	
Ngwerere	100 (20.4)	2 (2)	98 (98)	
Luiimba	34 (6.9)	0	34 (100)	
Chongwe	33 (6.7)	0	33 (100)	<0.001
Study location				
Urban	323 (65.9)	48 (14.9)	275 (85.1)	
Rural	167 (34.1)	2 (1.2)	165 (98.8)	<0.001
*Residence				
Low	73 (14.9)	2 (2.7)	71 (97.3)	
Medium	230 (46.9)	7 (3.0)	223 (96.9)	
High	187 (38.2)	41 (21.9)	146 (78.1)	<0.001
*Father's level of Education				
Primary	67 (13.7)	4 (5.9)	63 (94.0)	
Secondary	265 (54.1)	38 (14.3)	227 (85.7)	
Tertiary	158 (32.2)	8 (5.1)	150 (94.9)	0.004
Mother's level of Education				
Primary	147 (30.1)	19 (12.9)	128 (87.1)	
Secondary	235 (48.1)	24 (10.2)	211 (89.8)	
Tertiary	107 (21.8)	7 (6.5)	100 (93.5)	0.03
Treatment of water				
Not boiling				
Boiling	296 (60.5)	39 (13.2)	257 (86.8)	
	193 (39.5)	11 (5.7)	182 (94.3)	0.004
Keeping animals				
No	359 (73.4)	43 (11.9)	316 (88.0)	
Yes	130 (26.6)	7 (5.4)	123 (94.6)	0.034

4.4: Multivariable analysis of risk factors of *Cryptosporidium* infection

In the multiple regression analysis, residency and water treatment were the only significant independent predictors of *Cryptosporidium* infection in the presence of the other variables. Children from high density residential areas were found to be 10 times more likely to have *Cryptosporidium* infection (adjusted odds radio [AOR] =10.20; 95% CI: 2.28-45.66; p=0.002) compared to their counterparts from low density areas. Similarly, those who reported not to boil their drinking water were likely to have *Cryptosporidium* infection (AOR=0.74; 95% CI: 0.350-1.58; p=0.439), compared to those who were boiling water. (Table 4.4).

Table 4.4: Multivariable analysis of risk factors of *Cryptosporidium* infection

Characteristics	AOR	95% CI	P-value
Age	0.59	(0.96-1.02)	0.367
Study location			
Urban	Ref		
Rural	0.14	(0.307-0.624)	0.010
Residence			
Low	Ref		
Medium	2.27	(0.44-11.73)	0.326
High	10.20	(2.28-45.66)	0.002
Water treatment			
Not boiling	0.74	(0.35-1.58)	0.439
Boiling	Ref		
Keeping animals			
No	1.08	(0.43-2.72)	0.866
Yes	Ref		

COR=Crude odds ratios, AOR=Adjusted odds ratios, CI= Confidence interval, Ref=reference category

4.5: DNA amplification of *GP60* gene

All the 50 samples that were positive on Modified Ziehl Neelsen stain were genotyped by nested Polymerase Chain Reaction (nested PCR). Only sixteen from 50 positive samples gave a good sequence analysis at *gp60* gene. No amplification was observed in the negative controls. Figure 4.1 indicates amplification products on agarose gel.

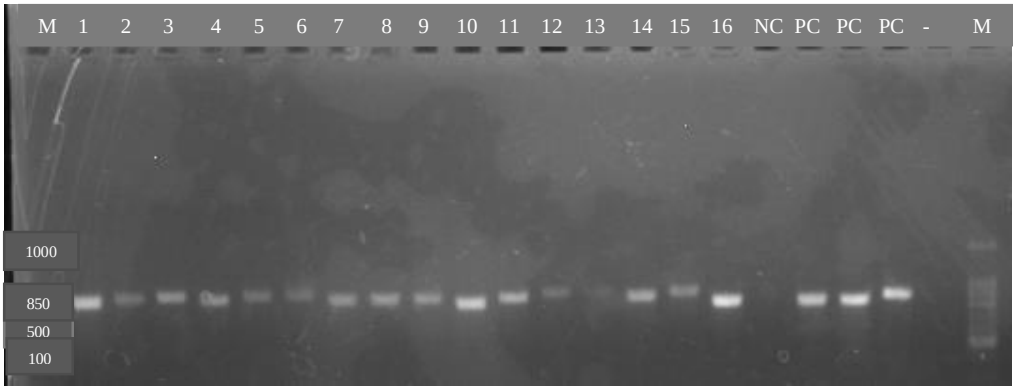


Fig 4.1: DNA amplification of *Cryptosporidium* species

The above fig 4.1, shows the secondary PCR products of *Cryptosporidium* after running agarose gel (1%) electrophoresis. Lane M 100 -bp DNA Marker; 850 bp Lanes 1-16 the products of PCR for DNA extracted samples; Lane NC: negative control and Lane PC: Positive control.

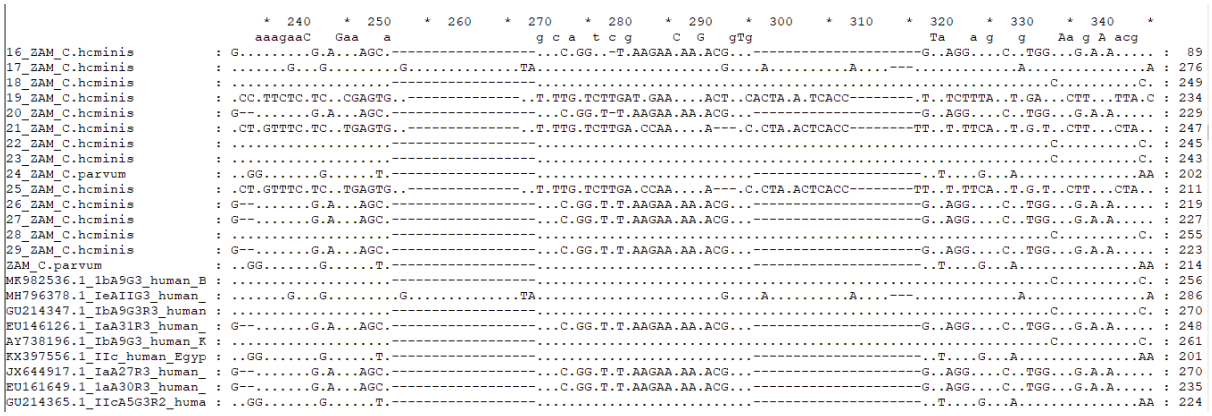
4.6: Species and genotype of *Cryptosporidium* isolates

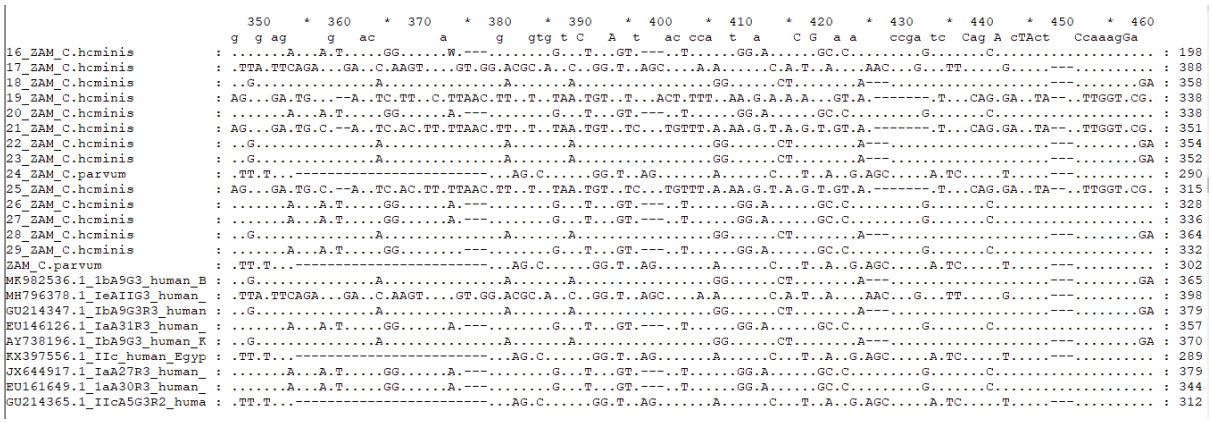
Of the total of 50 positive samples, high quality amplification of the *gp60* gene was obtained from 16 samples (2 rural, 14 urban). Both the rural isolates and 12 of the urban isolates were *C. hominis*; only 2 (urban) isolates were *C. parvum* and these were both anthroponotic genotype IIc. Subtype family Ia was isolated from a child from rural, IaA27R3, IaA30R3 and IaA31R3 were from urban. The most common subtype family identified (IbA9G3) was also identified in another child in rural areas (Table 4.5).

Table 4.5: Species and genotypes of *Cryptosporidium* isolates

Species	Subtype families	No. of Subtypes	Subtype distribution
C. hominis	Ia	Ia	1
		IaA27R3	1
		IaA30R3	1
		IaA31R3	3
	Ib	IbA9G3R2	2
		IbA9G3	5
	Ie	IeA11G3	1
C. parvum	IIc	IIcA5G3a	1
		IIcA5G3R2	1

Fig 4.2 below shows the variations in nucleotide sequences among the various *Cryptosporidium* families isolated in this study. Sequences of the *gp60* gene of *C. hominis* and *C. parvum* in this study (accession numbers MZ351216-MZ351230) were compared with *C. hominis* and *C.parvum* previous published sequences from GenBank, *C. hominis* JX644917 (Nigeria), EU161649 (UK), EU146128 (Kenya), AY736196 (Kuwait),GU214347 (UK), MK982536 (Bangladesh) and *C. parvum* MH796378 (Nertherland) and KX397556 (Egypt) (figure 2 below). There was a relationship between sequences of *Cryptosporidium* species in this study and other previous published sequences.(e. g. ZAM 17 sequence has the same sequence with MH796378 (Netherland), ZAM 24 was similar to JX397556 (Egypt) and ZAM 29,27,26,20,16 sequences were similar to those from Kenya, Nigeria and UK (EU146126, JX644917and EU161649) respectively.





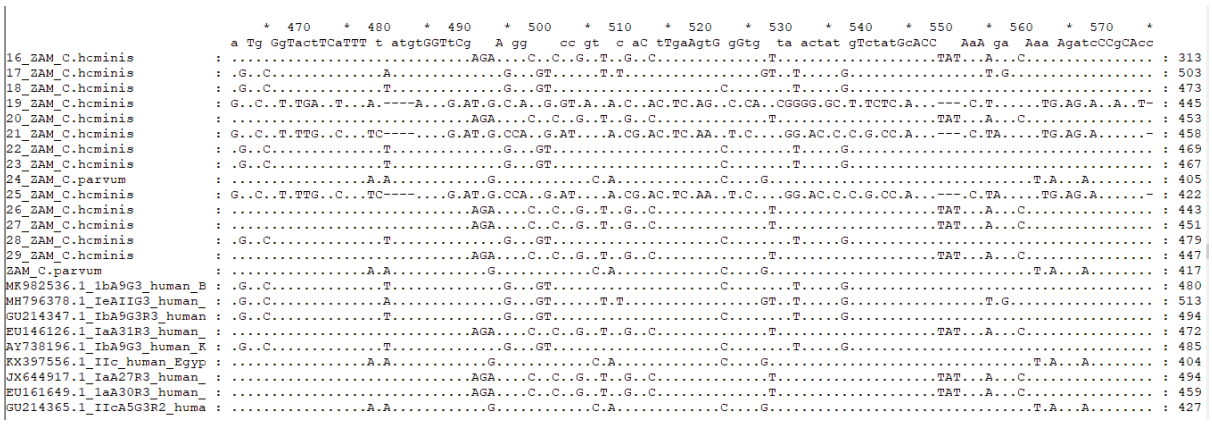


Fig 4.2 Alignment of nucleotide sequences of *Cryptosporidium* species

Phylogenetic tree was constructed using phylogenetic analysis containg reference genotypes of *C. hominis* and *C. parvum* obtained from previously published sequences from GenBank. Phylogenetic analysis of *Cryptosporidium* isolates revealed that the isolates were genetically diverse and did not constitute a geographically isolated clade being similar to what has been isolated elsewhere. The other 34 sequences were not included in further analysis as the sequences are short following alignment.

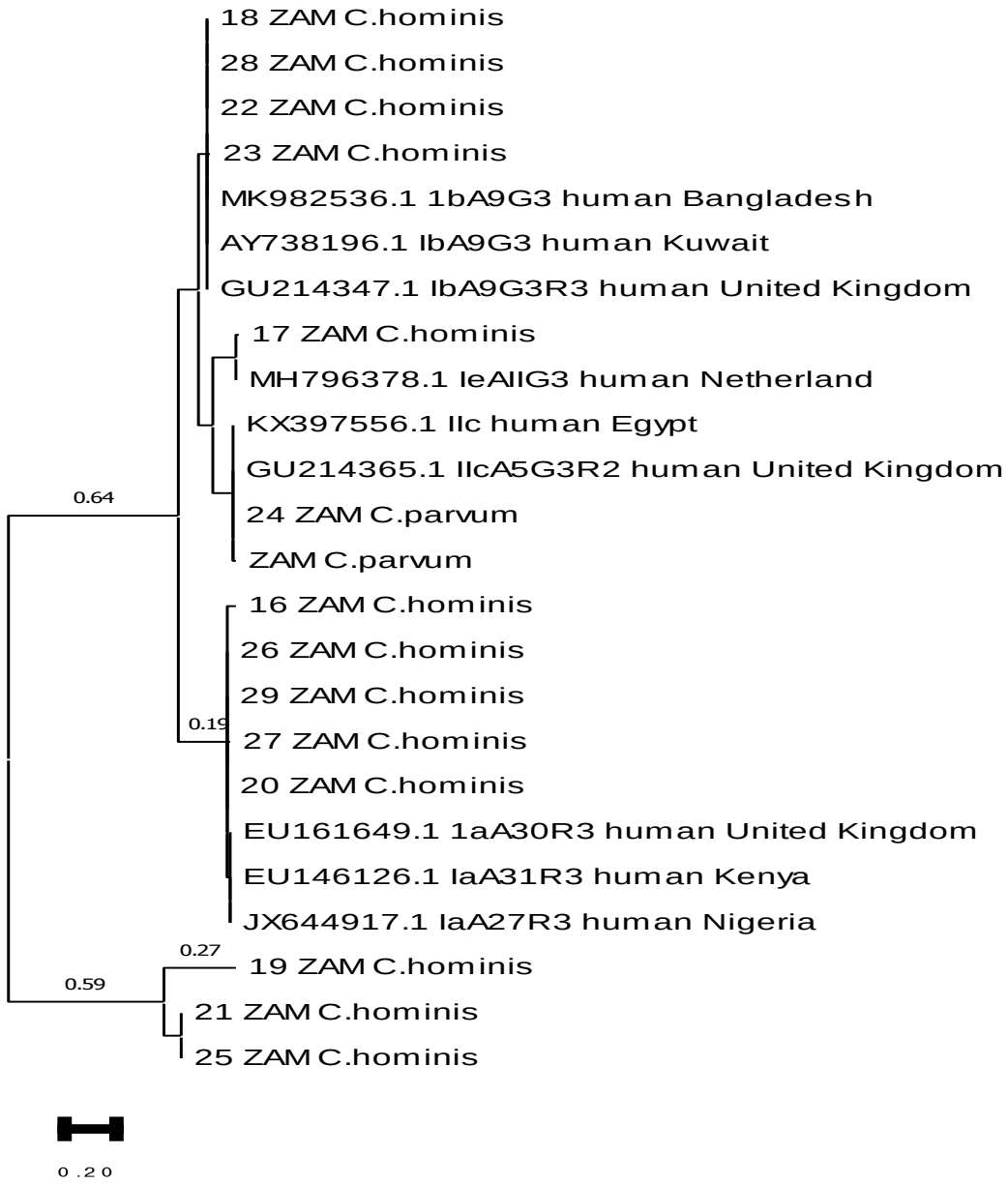


Figure 4.3: Phylogenetic analysis of *Cryptosporidium* species and subfamilies. The evolutionary history was inferred using the Neighbor-joining method (Saiton *et al.*,

1987). The tree was drawn to scale with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the Phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likehood method (Tamara *et al*., 2004) and are in the units of the number of base substitutions per site. This analysis involved 24 nucleotide sequences. Codon positions included were 1^{st} +2^{rd} + 3^{rd} + noncoding. All ambiguous positions were removed for each sequence per (pairwise deletion option). There were a total of 1077 position in the final dataset. Evolutionary analysis were conducted in MEGA 11 (Tamara *et al*., 2021).

NB less than 0.1 values were not shown in the tree.

CHAPTER 5: DISCUSSION

This study reports a *Cryptosporidium* health facility prevalence of 10.2% (95% CI: 7.8-13.2) in children under five years of age. Previous Zambian studies (both community based) have reported slightly higher prevalence at 30.7% (Siwila *et al.*, 2011); 18% (Nchito *et al.*, 1998)., consistent with studies in Egypt which found the prevalence at 60% and Cote d'Ivoire at 37% in children with diarrhoea (Abdel-Hafeez *et al.*, 2012; Koffi *et al.*, 2014). However, in another Egyptian study, low prevalence of 1.4% was reported in the tested children (Naquib *et al.*, 2018). The discrepancies in the reported prevalence of other studies and the current study could be due to the difference in the age of study population, study sites, environmental, behaviour and socioeconomic factors, as well as differences in timing in sample collection (Lal *et al.*, 2015). The other factor is that the current study used microscopy method to detect *Cryptosporidium* infection compared to previous studies which used Immunofluorescence and PCR for the detection of *Cryptosporidium* infection (Siwila *et al*., 2007 (prevalence was 6%), Siwila *et al*., 2011).

The results of the current study are comparable with those reported in under five children in Pakistan (10.9%) (Haider *et al*., 2012). It is evident that, worldwide, *Cryptosporidium* infection rates in children vary considerably, due to many factors such as seasonal variations, sanitation hygiene and health education. . In developed countries, the prevalence rate ranges from 0.1-9.1% (Fletcher *et al*., 2012), while in some developing countries the prevalence ranges from 2.98-25.9 % (Snelling *et al*., 2007).

The children enrolled in this study were largely resident in high- density housing. High density residential areas in developing countries such as Zambia are characterised by poor sanitation, rationed water supply and increased human -to -human interaction which contribute to increased risk of infection (Korpe *et al.*, 2019). Open defaecation by children also occurs. This finding is in agreement with a study in Bangladesh which reported person –person transmission as the major source (Korpe *et al.*, 2019). Studies published elsewhere (Palock *et al.*, 2010, Abal-febeiro *et al.*, 2015), found that residing in remote or rural regions was a risk factor for cryptosporidiosis. This was not the case in the current study. Very few samples were collected from the rural areas which makes it difficult to draw conclusions. However, an Iranian study (Ranjbar-Bahadori *et al*.,

2011) did not find differences in infection between children from rural and urban settings.

In most studies the prevalence rate of *Cryptosporidium* infections between males and females varies (AL-Shamini *et al.,* 2010). In this present study, *Cryptosporidium* infection was similar with other studies where infection rate was higher in male than in female children. This could be due to more frequent exposure of male children to gardens and farms, or differences in their behaviour (Eibach *et al.,* 2015, Taha *et al*., 2018). This is in contrast with the study in Palestine done by Al-Hindi *et al*. (2007), which recorded a higher incidence in females than in males, the authors attributed this to females being more exposed to source of infection than males.

Since age was not normally distributed in this study, the age was compared between those children from urban and rural areas. Children from urban were more likely to have *Cryptosporidium* infection than their rural counterparts. Previous studies have also observed *Cryptosporidium* infection in children less than two years of age (Ahmed *et al*., 2016, Kotloff *et al*., 2013, Squire *et al.,* 2017). High incidence of infection in under five children may be due to lack of pre-existing immunity and direct contact with human or animal faeces when crawling. Moreover, children are more exposed to water during playing, increasing the chance of getting infected. Also, children this age group (under five) are learning to be independent and therefore more vulnerable to acquire infection due to unhygienic behaviours (Al-Mohammed *et al*., 2010, Usfar *et al*., 2010, Checkly *et al.,* 2015, Anejo- Okopi *et al.,* 2016). Age was also found to be the risk factor for the occurrence of *Cryptosporidium* in the study by Khan *et al.* (2017).

Cryptosporidium infection was associated with failure to boil drinking water. *Cryptosporidium* infection has been correlated with the type of water used for consumption per household (Kelly *et al*., 1997; Dabirzadeh *et al.,* 2017). Many studies have emphasized that *Cryptosporidium* species is mostly found in untreated water (Dennely *et al.,* 2011). However, other studies have reported that children who were drinking water directly from the tap were at risk of having cryptosporidiosis, which may reflect/confirm the fact that chlorination of municipal drinking water does not inactivate oocysts (Al-Warid *et al.,* 2012, Quihui-Cota *et al.,* 2017, El-Badry *et al.,*

2017). Khalifa *et al.* (2014) showed that children who were coming from households where water was not treated were more likely to have *Cryptosporidium* infection.

Different methods for the detection of *Cryptosporidium* infection are used globally. These include microscopy, enzyme immunoassay, and Polymerase Chain Reaction (PCR) (Ghaffari *et al*., 2014, Chalmer *et al*., 2013, Khurana *et al*., 2018). Microscopy is the most widely used method in the diagnosis of cryptosporidiosis. The microscopic test requires special acid-fast staining with sensitivity of 56-75.4% Chalmer *et al*., 2011, Stark *et al.*, 2011). Although microscopy is used in most studies, it is a time-consuming procedure, tedious and requires experienced microscopist for parasite identification. Many researchers worldwide are using molecular methods due to their high sensitivity and specificity (100%) for identifying *Cryptosporidium* species and genotypes in faecal samples (Salyer *et al*., 2012, Moore *et al.*, 2016), but it requires trained personnel and well-equipped laboratory (Okangba *et al*., 2010). Immunoassays also play a vital role in the diagnosis of *Cryptosporidium* infections because of their excellent sensitivity (90%) and specificity (95%) (McHardy *et al*., 2014, Wasike *et al*., 2015).

Molecular characterisation of the isolates identified *C. hominis* as the predominant species (87.5%), *C. parvum* was only identified in two (12.5%) samples. Similar findings have been reported by Mulunda *et al*. (2020), El-Badry *et al.* (2015) and Ghallab *et al.* (2018) in diarrhoeic children. Of the six *C. hominis* subtype families described to date, the study identified three common subtypes including Ia, Ib and Ie. These subtype families have been reported in children in a number of developing countries (as reviewed by Sow *et al.*, 2016; Squire *et al.*, 2017). Within the subtype family, subtype 1b has been especially associated with abdominal pain, vomiting and diarrhoea (Chalmer *et al.*, 2010, Jex and Gasser 2010; Segura *et al.*, 2015). This study identified nine different subtype families (Table 4.6) with subtype IbA9G3 being the most frequent. In a recent Zambian study conducted by Mulunda *et al.*, (2020), *C. hominis* accounted for majority of the cases, with four subtype families (Ia, Ib, Id, and Ie), IeA113T3 being predominant. However, in that study the urban/rural source of the isolates was not ascertained. .The zoonotically transmitted *C. parvum* subtype families IIa, IId, IIe, IIl were not identified in this study, and only subtype family IIc was detected from two children. The subtype family IIc has previously been detected in human samples and its anthroponotic transmission has also been confirmed in the

United States, Canada, Europe and Australia (Xiao 2010). This subtype family was also found to be dominant in the samples from Nigerian children (Molloy *et al.*, 2010).

The study had limitations. First, because only one stool sample was submitted for investigation by each participant, this could have led to underestimating the prevalence of *Cryptosporidium.* Second, initial detection using microscopy, known to be insensitive would underestimate the overall prevalence among diarrhoea cases. Third, the study used the *gp60* gene only for genotyping *Cryptosporidium* species which is only useful for *C. hominis* and *C. parvum.* Lastly, HIV status was not determined so that the infection rate in both children who are immunosuppressed and immunocompetent could be compared.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

In conclusion, this study reports a cryptosporidiosis prevalence of 10.2% in children with diarrhoea with the peak in March that corresponds to the rainy season in Zambia. Children who came from high residential areas and households where drinking water was not boiled were more likely to have *Cryptosporidium* infection. Sequence analysis of the *gp60* gene from 16 positive samples (14 from urban and 2 from rural sources) revealed *C. hominis* (14/16) and *C. parvum* (2/16) and suggest that anthroponotic transmission is dominant.

6.2 Recommendations

- Households to boil their drinking water to reduce the spread of *Cryptosporidium* infections.
- Future studies to use PCR probably alongside Modified Ziehl Neelsen which is useful for identifying heavy infections.
- A longitudinal study to be done in future to have a better understanding of the molecular epidemiology of *Cryptosporidium* species and subtypes among children in urban and rural settings.

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8.0 APPENDICES

APPENDIX A: ETHICS CLEARANCE LETTER

THE UNIVERSITY OF ZAMBIA

BIOMEDICAL RESEARCH ETHICS COMMITTEE

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1st February, 2018.

Your Ref: 002-11-17.

Ms. Barbara Banda,
University Teaching Hospital,
Department of Pathology and Microbiology,
Parasitology Laboratory,
P/Bag RW 1X,
Lusaka.

Dear Ms. Banda,

RE: RESUBMITTED RESEARCH PROPOSAL: "CHARACTERISATION OF CRYPTOSPORIDIUM SPECIES FROM CHILDREN IN RURAL AND URBAN SETTINGS OF ZAMBIA" (REF. No. 002-11-17)

The above-mentioned research proposal was presented to the Biomedical Research Ethics Committee meeting on 31st January 2018. 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).
- Where appropriate, apply in writing to National Health Research Authority for permission before you embark on the study.
- **Ensure that a final copy of the results is submitted to this Committee.**

Yours sincerely,

Dr. S. H Nzala PhD
VICE-CHAIRPERSON

Date of approval: 1st February, 2018. **Date of expiry**: 31st January, 2019.

APPENDIX B: PERMISSION LETTERS

REPUBLIC OF ZAMBIA
MINISTRY OF HEALTH
UNIVERSITY TEACHING HOSPITALS
01 MAR 2018
SENIOR MEDIAL SUPERITENDANT
LUSAKA CHILDREN'S HOSPITAL
PRIVATE BAG RW1X, LUSAKA

MINISTRY OF HEALTH
University Teaching Hospitals
CHILDREN'S HOSPITAL

P/ Bag RW IX Lusaka, Zambia

Tel 254965/250305

OFFICE OF THE SENIOR MEDICAL SUPERINTENDENT

1st March, 2018

Ms Barbara Banda
University Teaching Hospital
Department of pathology & Microbiology
Parasitological Laboratory
P/Bag RW1X
LUSAKA

Dear Ms Banda.

RE: PERMISSION TO CONDUCT RESEARCH AT THE CHILDREN'S HOSPITAL.

Reference is made to your regarding the above captioned.

We note that you have been cleared by Biomedical Research Ethics Committee to conduct this research and permission has been granted by the Children's Hospital Management.

Since your research does not interfere with patients care, the hospital has no objection and permission is therefore granted.

Yours faithfully,

Dr J. Musuku
SENIOR MEDICAL SUPERINTENDENT.

Cc: Head Clinical Care

Cc: Head of Hematology Dept,

REPUBLIC OF ZAMBIA
MINISTRY OF HEALTH
UNIVERSITY TEACHING HOSPITALS
01 MAR 2018
HEAD CLINICAL CARE
CHILDREN'S HOSPITAL
PRIVATE BAG RW1X, LUSAKA

All Correspondence should be addressed to the Provincial Medical Officer
Telephone: +260 211 256815
Telefax: +260 211 256814

In reply please quote:

File No. ....

REPUBLIC OF ZAMBIA

MINISTRY OF HEALTH

PROVINCIAL MEDICAL OFFICE
P.O. BOX 32573
LUSAKA

22.03.18
CC/DDHO/LAB
[illegible]

16th March 2018

Barbara Banda
School of Health Sciences
Department of Biomedical Sciences
University of Zambia
Lusaka

Re: Permission to conduct study at Chongwe and Kafue District Hospitals

Refer to the above subject matter.

My office is in receipt of your letter dated 13th March 2018 in which you are requesting for permission to conduct a study in Kafue at Kafue district hospital and at Chongwe district hospital in Chongwe district for your dissertation on the topic "Characterization of Cryptosporidium species from children in the rural and urban settings of Zambia".

I am glad to inform you that permission has been granted for you to conduct the study and kindly reveal us with your study proposal. However, during the period you will be conducting your research there must be minimum interruption to health service delivery in the targeted health facilities. Besides, the final report of your research you must also share with this office and the targeted districts hospitals where the study will be done.

Yours faithfully,

REPUBLIC OF ZAMBIA
MINISTRY OF HEALTH
PROVINCIAL HEALTH OFFICE
16 MAR 2018
PROVINCIAL HEALTH DIRECTOR
P.O. BOX 32573
LUSAKA

23/03/18
noted

29/08/18
To visit the hospital
Luimba and
Ngwerere HP
for data collection

Dr. Consity Mwale
Provincial Health Director
LUSAKA PROVINCE

cc. Lusaka District Health ~~Director – Chongwe~~
cc. Lusaka District Health Director – Kafue

APPENDIX C: INFORMATION SHEET-ENGLISH

Study Title: Characterisation of *Cryptosporidium* species from Children in Urban and Rural Settings of Zambia.

I am Barbara Banda, a Master of Science student at the University of Zambia in the School of Health Sciences, conducting a study on Cryptosporidiosis in children. The information collected will be used for academic purposes only.

Cryptosporidiosis is a diarrhoeal disease caused by a parasite, *Cryptosporidium*. It causes diarrhoea in both humans and animals.

The purpose of this study is to identify species or strains that cause infection in children and identify associated risk factors.

The study will provide information not only in *Cryptosporidium* species but also on the mechanism of spread, distribution and transmission pattern. This will form a basis for formulating prevention and control measures.

Participation in the study is completely voluntary and you may refuse to have your child / dependent participate. There will be no risk to your child / dependent.

CONTACT NUMBERS:

In case of any queries contact the following numbers:

1. Barbara Banda (Principal Investigator) Cell: 0977370296

2. Professor Paul Kelly (Principal Supervisor) Cell: +260 966 75185

3. Dr Joyce S. Saasa (Co-Supervisor) Cell: 0977319826

APPENDIX D: INFORMATION SHEET-TONGA

Study Title: Characterisation of *Cryptosporidium* species from Children in Urban and Rural Settings of Zambia.

Ndime Barbara Banda, ndiya kuba habupampu kucikolo cipati caku University of Zambia mu cikolo ciisya zya nseba, tuvuntauzya zya Cryptosporidiosis mubana basyoonto.Twaambo ntotu bwezelela tuyoobelesya kukuiisya kwalo kwamana.

Cryptosporidiosis mbulwazi buletwa akauka kaiitwa kuti *Cryptosporidium*. Kapa bulwazi bwakusoomona kubantu alimwi aku banyama.

Cipati kuli kuvuntauzya ooku nkuti tubone tumwi tukauka tupa nokuba zyimwi zyipa kuyambukila ku bana alimwi aku jana nzila zyimwi zyinga zyilaleta ntenda.

Kukuvuntauzya ooku kuyoo tondezya kutalibuyo makani aatuka twa *Cryptosporidium* kwalope, pele nkubona mbuli bwende mbokayanda alimwi anzila mbokayambukila. Eeci ciyoo tondezya nzila yakukwabilila akugwasyilizya kubika nzila zyilagwasya kwiimika.

Kutola lubazu kukuvuntauzya ooku nkwakulipa alimwi inga mwasala kulesya mwana wenu naba mwana ngomulela kutola lubazu.

Naa kuti mwabaa mibuzyo inga mwatuma luwaile ku manamba aya.

1. Barbara Banda (Principal Investigator) Cell: 0977 370296

2. Professor Paul Kelly (Principal Supervisor) Cell: +260 966 75185

3. Dr Joyce S. Saasa (Co-Supervisor) Cell: 0977 319826

APPENDIX E: INFORMATION SHEET-BEMBA

Study Title: Characterisation of *Cryptosporidium* species from Children in Urban and Rural Settings of Zambia.

Ishina lyandi ninebo Barbara Banda, umwana we sukulu usambilila ku sukulu likalamba ilya University of Zambia, ukusambilila aba fikukile sakwe mukupima ifya bumi. Tulipakufwailikisha pafya tushishi twa Cryptosporidiosis mu bana abanono. Ifyo tukasanga muli ilisambililo fyika bofyewa kumasambililo fye capwa.

Cryptosporidiosis bulwele bwakupolomya ubuletwa nakashishi aketwa ati *Cryptosporidium*. Aka kashishi kalaleta ubulwele bwa kupolomya kubantu elyo nakufinama.

Ichi kalamba ichili mukufwailikisha uku, kumona ifyo utushishi nangula ukumona ifilenga ukwambukila mubana pamo nefingalenga ukwambukilwa.

Ukufwailikisha ukuli muli ilisambililo takwakapele ukwishiba pafya tushishi twa *Cryptosporidium* tweka fye iyo, ukufyailikisha uku kukalenga ukumona inshila iyo tusabankaninamo, ifyo tutanda kabili neshila eyo twambukila. Ichi chikalenga ukukwata inshila isha kuichingilila neshila ishafwilisha ukulesha ukwambukilwa.

Uku sendamo ulubali muli ilisambililo, kabili kuti mwakanywa ukusuminisha umwana wenu nangula kuomusunga ukusendamo ulubali.Takwakabe ubwafya ubu kaponamo mu mwana wenu nangula muomusunga.

Nga chakuti mwinga kwata amepusho kuti mwatuma ku ma namba aya.

1. Barbara Banda (Principal Investigator) Cell: 0977 370296
2. Professor Paul Kelly (Principal Supervisor) Cell: +260 966 75185
3. Dr Joyce S. Saasa (Co-Supervisor) Cell: 0977 319826

APPENDIX F: INFORMATION SHEET –NYANJA

Study Title: Characterisation of *Cryptosporidium* species from Children in Urban and Rural Settings of Zambia.

Dzina langa ndine, Barbara Banda nditengako mbali muma phunzilo akuya la University of Zambia muchigayo za zaumoyo, ndiku fufuza paza Cryptosporidiosis mu ana achichepele. Ndemanga zotuluka muchipunzitso ziza gwilitsidwa nchito zamaphuzilo chabe.

Cryptosporidiosis ndi matenda yomwe yabwela ndi kalombo ko chedwa, *Cryptosporidium*. Kama bwelesa matenda yo tulula ku bantu ndi nyama zomwe.

Cholinga chimphunziso chi ndiku funa kuzhiba nditulombo totani ndiponso ndi njila zotani zomwe ana angate kutengemo matenda aya ndi makakalidwe omwe anga falise matendaya.

Muchiphunzisochi muzachoka ndemanga yokuza kosati za tudoyotu toka twa *Cryptosporidium* chabe ayi komanso ndingila yomwe yofalisidwilamo ndi matengedwe yamatendaya. Ndemangai izatandizila kupeza njila yazotetezela ndikugonjeza kwa matenda aaya.

Kutengako mba muchi phuzisoci ndi mozipeleka, mungate kulesamwana wanuyo kapena osungudwa kukana kutengako mbali. Palibe koipa kena kali konse kangate kuchi ku mwana wanuyo kapena osungidwayo.

Ngati muli ndi mafunso mungate kutuma lamya kuli aa s:

1. Barbara Banda (Principal Investigator) Cell: 0977 370296
2. Professor Paul Kelly (Principal Supervisor) Cell: +260 966 75185
3. Dr Joyce S. Saasa (Co-Supervisor) Cell: 0977 319826

APPENDIX G: QUESTIONNAIRE -ENGLISH

STUDY TITLE: CHARACTERISATION OF *CRYPTOSPORIDIUM* SPECIES FROM CHILDREN IN URBAN AND RURAL SETTINGS OF ZAMBIA

INSTRUCTIONS

Answer the all questions in this questionnaire.

Name of the: Province……..

District……

Health facility…….

Demographic information:

1. Serial Number……..
2. Name……
3. Sex……
4. Age……...
5. Residence…….
6. House number……..
7. Level of Education of Father (Primary / Secondary / Tertially).
8. Level of Education of Mother (Primary / Secondary / Tertially).
9. How many members in the family? .....
10. Do your parents/guardian work? Yes / No.
11. What is the source of income? .....
12. Which of these animals are near your home? Dogs / Cats / Goats / Cattle.
13. Do you treat your drinking water? Chlorination / boiling.
14. Does your child clean his/her hands before eating? Yes / No.
15. Does your child wash hands after using the toilet? Yes / No.
16. Has this child suffered from any diarrhoeal diseases recently? Yes / No.

APPENDIX H: QUESTIONNAIRE-NYANJA

STUDY TITLE: CHARACTERISATION OF *CRYPTOSPORIDIUM* SPECIES FROM CHILDREN IN URBAN AND RURAL SETTINGS OF ZAMBIA

INSTRUCTIONS

Yankhani mafunso onse omwe ali pomwepa.

Dzina la dera……

Boma…….

chipatala……

Malo m`mene mukhala anthu:

1. nambala yapadera……..

2. dzina …….

3. mkazi/mwamuna……...

4. zaka……...

5. malo……..

6. nambala ya nyumba……..

7. maphunzilo omwe kholo la chimuna linakwanitsa (Pulaimale / Sekondale / apamwamba).

8. maphunzilo a kholo la chikazi inakwanitsa (Pulaimale / Sekondale / apamwamba).

9. kodi ndi angati ali mbanja? .....

10. kodi makholo/okusunganiagwila nchito? inde / ai.

11. nkuti komwe achotsa ndalama zowathandiza? .....

12. pa ziweto izi ndi zotani zimene zipezeka pafupi ndi inuyo? agalu / achona / mbuzi/ng`ombe.

13. kodi mumaikako mankhwala kumadzi anu akumwa? Chlorine / kugadutsa.

14. kodi mwana wanu amasamba kumanja akalibe kudya zakudya? inde/ ai.

15. nanga mwana wanu amsamba kumanja pambuyo pogwilitsa nchito chimbuzi? inde / ai.

16. kodi mwana uyu anadwalapo matenda otsegula m`mimba kumbuyoku posachedwapa? inde/ ai.

APPENDIX I: QUESTIONNAIRE-BEMBA

STUDY TITLE: CHARACTERISATION OF *CRYPTOSPORIDIUM* SPECIES FROM CHILDREN IN URBAN AND RURAL SETTINGS OF ZAMBIA.

INSTRUCTIONS

Yasukeni amepusho yonse.

Ishina lya chitungu……...

Iboma……..

Icipatala……...

Imikalile ya banthu

1. Inambala yaibela…….
2. Ishina……...
3. Icifyalilwa……...
4. Imyaka…….
5. Uko mwikala……
6. Inambala ya ng`anda……
7. Apafika abawishi mumasambililo……
8. Apafika manyina mumasambililo……
9. Mwaba banga mulupwa? .....
10. Abafyashi abamusunga, bushe balabomba? Ee/awe.....
11. Nikwisa bafumya indalama? ……
12. Ninamanshi ishaba mupepi nokomwikalila pali ishi? imbwa/ba pushi/ imbushi/ing`ombe
13. Bushe mulabika umuti mumenshi munwa?tulabomfya chlorine/ tulabilaushako
14. Bushe umwana wenu alasamba kuminwe apo talalya? Ee/awe
15. Bushe umwana wenu alasamba kuminwe panuma yakubomfya ichimbusu? Eee/awe
16. Bushe uyu mwana alilwalapo ubulwele bwakupolomya nombaline? Eee/awe

APPENDIX J: QUESTIONNAIRE-TONGA

STUDY TITLE: CHARACTERISATION OF *CRYPTOSPORIDIUM* SPECIES FROM CHILDREN IN URBAN AND RURAL SETTINGS OF ZAMBIA.

INSTRUCTIONS

Amuvwiile mibuzyo eeyi.

Izina lya cilawo…….

Ku cooko……

Cibbaddela…….

Mbola kala bantu

1 .Mweelwe…….

2. Izina…….

3. Musankwa/Musimbi……..

4. Myaka yaku zyalwa……....

5. Busena kukalwa…….

6. Inamba ya Ng'anda……

7. Cigga calwiyo lwa baushi ku Primary/ ku Secondary/lwiiyo lupati.

8. Cigga calwiyo lwa banyina ku Primary/ku Secondary/lwiiyo luati

9. Mweelwe waba bantu bali mumukwashi?

10. Sena bazyali naa bamulela kuli milimo njobabeleka? Iiyi/ Pepe

11. Ino nincito nzi iikutaukwa iimuletela bulumbu?

12.Ino mbanyama nzi alibaaba ibajanywa munsimunsi ang'anda yenu? Babwa/Tukiti/Mpongo/Ng'ombe.

13. Sena meenda akunywa mulaa bika musamu nokuba kwaajika? Clorination/kwajika.

14. Sena mwana wenu ulalisalazya katana talika kulya? Iiyi/Pepe.

15. Sena mwana wenu ulasamba kumaanza amana kubelesya cimbuzi? Iiyi/Pepe.

16. Sena kuli ciindi mwana ooyu naka cisidwe bulyazi bwa ku soomona cainoino? Iiyi/Pepe.

Contact Details

1. Barbara Banda (Principal Investigator)

   Cell: 0977370296

   email address: barbarabanda243@yahoo.com

   The University of Zambia,

   School of Health Sciences,

   Department of Biomedical Sciences,

   P.O Box 50110, Lusaka.

2. Professor Paul Kelly (Principal Supervisor)

   TROPGAN group

   Department of Internal Medicine,

   University of Zambia School of Medicine,

   Nationalist Road, Lusaka, Zambia.

   Cell: +260 966 751875

   email address: m.p.kelly@qmul.ac.uk

3. Dr Joyce Siwila Saasa (Co-Supervisor)

   School of Veterinary Medicine,

   University of Zambia, Lusaka

   Cell: 0977319826

APPENDIX K: CONSENT FORM

PARENT/GUARDIAN CONSENT

I confirm that I understand the information for this study.

I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.

I understand that my child / dependent's participation is voluntary and that I am free to withdraw my child / dependent any time, without giving any reason, without my medical care or legal rights being affected.

I agree that my child / dependent can take part in the study.

Please sign your name or put your thumb print

Name of the child…….

Date……..

Witness: Name…….

Signature……...

Date……..

APPENDIX L: DATA COLLECTION TOOL/FORM

To be completed by the Principal Investigator

Name……

ID number……

Sex……

Age……

Area of residence……..

1. **Stool Examination**

   Macroscopic: Appearance…….

   Consistency……..

   Microscopy: Formal ether Concentration……

   Modified Ziehl Neelsen Stain:

	Present
Cryptosporidium oocyst	Yes/No

2. **Nested PCR /Sequencing**

   Genotype identified……..

   Subgenotype identified ……

APPENDIX M: Table 3.1 Dependent and independent variables

Dependent variable (outcome variable)	**Definition of variable**	**Scale of measure**
Having *Cryptosporidium*/ no *Cryptosporidium*	Yes /No	Categorical (Binary)
Independent variable (Predictor variable)	**Definition of variable**	**Scale of measure**
Sex	Male / Female	Categorical (Binary)
Location	Rural /Urban	Categorical (Binary)
Residence	Low, Medium, High	Categorical (Nominal)
Father's education	Primary, Secondary, Tertially	Categorical (Nominal)
Mother's education	Primary, Secondary, Tertially	Categorical (Nominal)
Father employed	Yes /No	Categorical (Binary)
Mother employed	Yes /No	Categorical (Binary)
Water treatment	Yes /No	Categorical (Binary)
Keeping animals	Yes /No	Categorical (Binary)