

**Intestinal protozoa causing diarrhoea in children
aged 0-5 years in the eThekweni district of
KwaZulu-Natal.**

Nakita Reddy

Submitted in fulfilment of the requirements for the degree of Master of Medical Science in the Department of Medical Microbiology: Infection, Prevention and Control. In the School of Laboratory Medicine and Medical Science, Nelson R Mandela School of Medicine, University of KwaZulu-Natal.

July 2016

Supervisor: Professor A. W. Sturm

Declaration

I, Nakita Reddy, declare that this dissertation is my own unaided work, which has not been submitted previously to this or any other University or Institute.

Assistance rendered for this study has been acknowledged. The work of other researchers used in this dissertation has been duly noted.

This study was carried out at the Medical Microbiology – Infection, Prevention and Control Laboratory of the University of KwaZulu-Natal, under the supervision of Professor A.W. Sturm and co-supervisor, Dr Z. L. Kwitshana.

Ethical approval to conduct this study was obtained from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal.



Professor A.W. Sturm

(Supervisor)



Nakita Reddy

(Master's Candidate)

18 July 2016

**Dedicated to my parents, parents in-laws, husband and late
grandmother**

*For their continuous love, support and understanding throughout my
academic endeavours.*

Presentations

Parts of this dissertation were presented at the following conferences:

- 1) University of KwaZulu-Natal's College of Health Sciences Research Symposium,
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List of Acronyms

AIDS: Acquired Immune Deficiency Syndrome

BREC: Biomedical Research Ethics Committee

CDC: Centre for Disease Control and Prevention

CTAB: Cetyl Trimethyl Ammonium Bromide

DNA: Deoxyribonucleic acid

DNTP: Deoxynucleotide Triphosphate

EDTA: Ethylenediamine Tetra-acetic Acid

ELISA: Enzyme Linked Immunosorbent Assay

GIT: Gastrointestinal tract

HCl: Hydrochloric acid

HIV: Human Immunodeficiency Virus

KZN: KwaZulu-Natal

MgCl₂: Magnesium Chloride

MWM: Molecular weight marker

NaCl: Sodium chloride

NC: Negative control

ND: Not done

PCR: Polymerase Chain Reaction

PL1: Specimens found positive for *C. parvum* 1 in a private laboratory

PL2: Specimens found positive for *G. lamblia* 3 in a private laboratory

PL3: Specimens found positive for *C. parvum* 2 in a private laboratory

PL4: Specimens found positive for *G. lamblia* 2 in a private laboratory

S: Specimen

SDS: Sodium-dodecyl Sulphate

TE: Tris EDTA

T_m: Melting temperature

WHO: World Health Organisation

Units of Measure:

Base pairs: bp

Degrees Celsius: °C

Grams: g

Gravitational force: x g

Greater than: >

Less than: <

Micro gram: µg

Micro-litre: µl

Micro molar: µM

Millilitre: ml

Milli-molar: mM

Molar: M

Nanogram: ng

Percent: %

Significance: p

Units: U

Abstract

In Africa, diarrhoea accounts for up to 75% of all illnesses in children aged five years or younger. In KwaZulu-Natal scanty data exists on the prevalence of intestinal parasites causing diarrhoea in this age group. *Cryptosporidium parvum*, *Entamoeba histolytica* and *Giardia lamblia* are the most common pathogens known to cause diarrhoea in children. Approval to conduct this study was obtained from the University of KwaZulu-Natal's Biomedical Research Ethics Committee. Informed consent was obtained from parents or other caregivers. Stool specimens of 251 children were collected and preserved in 5% formalin. Faecal smears were prepared for microscopic examination and stained using the modified Ziehl-Neelsen stain and the modified Ryan-Blue trichrome stain. The modified Ziehl-Neelsen stain detected *G. lamblia* in 1% of the children and *C. parvum* in 15%. With the modified trichrome stain these numbers changed to 2.5% and 7% respectively. When the results of both staining techniques were combined the prevalence increased to 3% for *G. lamblia* and 21% for *C. parvum*. Our results show that diarrhoea amongst the population under investigation is mainly caused by *C. parvum*. PCR to confirm the number of *G. lamblia* and *C. parvum* obtained by microscopy was unsuccessful. Various DNA extraction methods were tried. The lack of success is likely the result of the resistance of the cyst wall to chemicals and temperature changes. This warrants further investigations.

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Abstract

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Chapter One: Introduction

Parasitism is a relationship between species where one species the parasite, benefits at the expense of the other, the host. This is not restricted to any group of organisms. However, historically the term “parasitology” has been used for the science that deals with helminths and protozoa. In this dissertation the term parasite refers to these two groups of organisms.

Infections caused by intestinal parasites are amongst the most common infections globally (Nxasana *et al.*, 2013). These include protozoal infections that cause diarrhoea. Diarrhoea is the leading cause of morbidity and mortality amongst children in the developing world (Tinuade *et al.*, 2006).

Diarrhoea is defined as the passage of three or more loose stools within a period of twenty four hours (WHO, 2007). Diarrhoea can be categorized based on the duration, as acute or persistent (chronic). If the diarrheal episodes last for less than fourteen days, it is termed acute diarrhoea. If the episodes last for more than fourteen days it is termed persistent diarrhoea (Abba *et al.*, 2008). Such chronic diarrhoea often occurs in immune-compromised individuals, such as patients with AIDS.

In Africa, an estimated 25-75% of all illnesses in children under the age of five are due to diarrhoea (Kirkwood, 1991). In developing countries parasitic disease resulting in

diarrhoea, remains a serious health problem (Akingbade *et al.*, 2013). Nearly 90% of diarrhoea is due to infections from unsafe drinking water, poor hygiene and inadequate sanitation (Godana and Mengistie, 2013).

In developing countries the mortality rate as a result of diarrheal infections is as high as 56% (Samie *et al.*, 2012). Annually, diarrhoea contributes to 4.6-6 million child deaths in Africa, America and Asia, of which 80% occur within the first two years of life (Ali *et al.*, 2009).

Throughout South Africa high levels of intestinal parasites have been reported. In Mthatha, in the Eastern Cape Province, more than 60 % of primary school children were found to have intestinal parasitic infections. The majority of these pupils carried multiple parasites (Nxasana *et al.*, 2013). A study conducted in the Vhembe district in the Limpopo province, investigated the causes of diarrhoea in pupils from two schools and patients attending three different hospitals. They found that 78% of those with diarrhoea were children aged less than five years. Intestinal parasites were detected in 68% of all specimens screened, indicative of a high prevalence in this community (Samie *et al.*, 2012).

In KwaZulu-Natal the reported prevalence for intestinal parasites among school children ranged between 70 – 100% (Schutte *et al.*, 1977, Evans *et al.*, 1987, Taylor *et al.*, 1995 and Mabaso *et al.*, 2003). A large proportion of the infected population live in rural and peri-

urban areas, in which there is inadequate supply of water and inadequate sanitation (Nxasana *et al.*, 2013). According to South African Statistics, 22.3 % of people live in informal settlements, traditional dwellings or other sub-optimal living conditions, and only 70% of the population have access to running water. In the eThekweni district of KwaZulu-Natal, only 60.2% have access to piped water within their dwelling (Statistics South Africa, 2008). With access to clean drinking water, implementation of flushed toilet facilities and practicing good hygiene, diarrhoea can be reduced significantly (Samie *et al.*, 2009).

In KwaZulu-Natal there is scanty data on the prevalence of intestinal parasites causing diarrhoea in children of five years of age and below. The lack of interest and expertise in the field of parasitology, often leads to under-diagnosis and misdiagnosis of parasitic infections (Mkhize-Kwitshana and Mabasa, 2012). In addition, more than one diagnostic test needs to be employed in order to optimise the accuracy and reduce the levels of misdiagnosis. This is laborious and time-consuming.

As diarrhoea alone kills more children than measles, malaria and AIDS combined (Godana and Mengistie, 2013), the aetiology of diarrhoea and the drug susceptibility profiles of these causative agents, including parasites needs to be monitored. This will lead to strategies for planning and implementing interventions, as well as the assessment of the effect of these interventions (Samie *et al.*, 2012).

In KwaZulu-Natal the mortality in infants in the 0-5 year age group is still high. Diarrhoea and respiratory tract infections are among the leading causes. This is despite the fact that vaccination coverage is high. This can be because microorganisms other than those covered by the vaccination program are causing the infections. This led us to investigate the aetiology of diarrhoea. This study presented here is therefore part of a bigger study. We aimed to determine the prevalence of intestinal parasites causing diarrhoea in children within the 0-5 year age group, in the eThekweni district of KZN. Microscopic analysis using two staining techniques was applied to this study. PCR was applied to this study with the purpose of verifying the results obtained from microscopic observations.

Chapter Two: Literature Review

2.1. Anatomy and Physiology of the Gastrointestinal Tract

The gastrointestinal tract (GIT) starts from the mouth where food and water is ingested and ends at the anus where waste products are expelled. The tract is divided into the upper GIT which extends from the mouth to the duodenum, the middle GIT continuing from the duodenum to the colon and the lower GIT extending from the colon to the anus. The upper, middle and lower GIT is also known as the foregut, midgut and hindgut, respectively (Kapoor, 2016). The middle and lower GIT is made up of the small and large intestine. The small intestine is differentiated into three components the duodenum, ileum and jejunum whilst the large intestine is made up of the caecum, ascending, transverse, descending and sigmoid colon, as well as the rectum (Reinus and Simon, 2014).

After food is ingested, it is chewed. Chewing stimulates the production of saliva which initiates the breakdown of food. Food particles are then passed down the oesophagus with the aid of mucus and peristaltic muscular movement to reach the stomach. The stomach controls the rate of entry of food and serves as a temporary location for its storage.

Preliminary digestion is facilitated by acid activated lipases and pepsin, resulting in chyme (Martini, 2001). In this process, microbes ingested with the food are killed in the acidic proteolytic mix.

When chyme enters the duodenum the pH changes from acidic to alkaline due to the addition of bile and pancreatic enzymes (Martini, 2001). The small intestine has a length of 8 m. Its surface area is enlarged due to the presence of villi and microvilli on the surface of

enterocytes (Reinus and Simon, 2014). Hence it is a site where the majority of the nutrients are absorbed (Carr and Toner, 1984). This is also the site where drug absorption takes place. The large intestine is where the absorption of water from the digested products occurs. Semi-solid faeces are passed to the rectum for excretion via the sphincter muscles of the anus (Martini, 2001).

The intestine is sterile at birth. However over a period of time more than a thousand different species of microbes populate the intestine. These organisms are both symbionts and commensals that exhibit a positive interaction with the intestine of the host (Lozupone *et al.*, 2012). Interactions facilitated by symbionts include digestion of nutrients and stimulation of the immune system. Of microbiological importance is the barrier mediated by symbionts, which prevents the dissemination of disease causing pathogens into other areas of the body that are sterile (Sekirov *et al.*, 2010).

2.2. Non-Infectious Causes of Diarrhoea

Although diarrhoea is predominantly caused by pathogens entering the GIT, there are a few causes of diarrhoea that are non-infectious. For instance an incorrect diet including carbohydrates such as lactose may result in diarrhoea (Fan and Sellin, 2009). Another cause could stem from the consumption of food poisoned with harmful chemicals (Esrey, 1990). Diarrhoea could also be a result of irritable bowel syndrome or intestinal diseases such as Crohn's disease, celiac disease, ulcerative colitis and inflammatory bowel disease. Medication used in the treatment of cancerous cells may lead to diarrhoea; use of antacids of magnesium origin may also cause symptoms of diarrhoea. (National Institute of

Diabetes and Digestive and Kidney Diseases, 2013). Another possible cause of gastroenteritis could be attributed to the malabsorption of bile salts (Fan and Sellin, 2009).

Broad spectrum antibiotics, such as tetracycline target both pathogens and commensals of the gastrointestinal tract. Eradication of the pathogen as well as the gut microflora, results in re-colonization by other bacteria such as *Staphylococcus aureus*. Such re-colonisation has been associated with enterocolitis and diarrhoea. Shortly after clindamycin was introduced as a therapeutic agent, findings demonstrated a blanket of fibrinous pseudomembrane covering the colonic mucosa. This resulted in severe diarrhoea. It was later shown that, clindamycin was not the cause. Instead clindamycin inhibited the normal gut flora which adversely resulted in multiplication of the clindamycin resistant *Clostridium difficile* and hence caused symptoms of diarrhoea (Goering *et al.*, 2008).

2.3. Infectious Causes of Diarrhoea

Infectious causes of diarrhoea include bacterial, viral and protozoal pathogens. Bacterial infectious agents include; *Campylobacter jejuni*, (Probert *et al.*, 2016) *Salmonella spp.*, *Shigella spp.*, (Mohammed, 2015) *Vibrio Cholera* (Rathur *et al.*, 2014) and *Clostridium difficile*, (Probert *et al.*, 2016). Bacterial culprits of diarrhoea also include; enteropathogenic, enterotoxigenic, enteroadherent as well as enterohaemorrhagic *Escherichia coli*. (Ogunsanya *et al.*, 1994). Other organisms like *Klebisella oxytoca*, *Pseudomonas aeruginosa* and *Proteus spp.* (Mohammed, 2015) have been associated with episodes of diarrhoea but their causal relationship is still up for debate.

Viruses such as *rotavirus*, *norovirus*, *astrovirus*, *adenovirus* and *saprovirus* cause infectious diarrhoea with a high prevalence among children under the age of five (Ren *et al.*, 2013). Protozoa such as *Cryptosporidium parvum*, *Entamoeba histolytica* and *Giardia lamblia* are considered important causes of diarrhoea (Verweij *et al.*, 2004).

2.4. Entero-Pathogenic Protozoa Associated with Diarrhoea

The differences in the degree of pathogenicity among gastrointestinal protozoa as well as the immune status of the infected host are illustrated in Table 2.1. Organisms are listed and discussed in alphabetical order.

Balantidium coli; is classified as a definite pathogen since it causes severe diarrhoea in immune compromised individuals by HIV infection. However, when present in the immune competent host *Balantidium coli* does not cause severe disease (Schuster and Ramirez-Avila, 2008).

Whether *Blastocystis hominis* is a pathogen or not remains debatable. Therefore in Table 2.1., it is classified as a probable pathogen. Studies however have identified a few subtypes (ST-1, ST-2, ST-4 and ST-8) of *Blastocystis hominis* associated with severe diarrhoea. However, additional studies could not confirm an association with pathogenicity. Higher detection rates of *Blastocystis hominis* has been associated with AIDS patients; indicating that immune compromised individuals may be more susceptible to this parasite (Roberts *et al.*, 2013).

Chilomastix mesnili is often detected in stool specimens together with other pathogenic protozoa, but it is not considered pathogenic. It is present in symptom free, immune competent hosts (El-Ammari and Nair, 2015). *Cyclospora cayetanensis* can definitely be categorised as a pathogen (Table 2.1.), as it has been associated with several outbreaks involving both immune suppressed and immune competent people (Chawla and Ichhpujani, 2011).

Dientamoeba fragilis was initially described as a harmless commensal of the gastrointestinal tract. Recent studies show the presence of *Dientamoeba fragilis* in stool specimens of patients suffering from diarrhoea. After treatment was administered to these patients, *Dientamoeba fragilis* was eradicated. Hence it is considered a possible pathogen. There have been studies that have reported *Dientamoeba fragilis* to be more common in the HIV infected population, whilst other studies indicate that it is more common in non-HIV infected individuals (Barratt *et al.*, 2011).

Endolimax nana (Santos *et al.*, 2014), *Entamoeba coli* (Rayan *et al.*, 2010), *Entamoeba hartmanni* (Becker *et al.*, 2011) and *Iodamoeba butschili* (Issa, 2014) have all been reported in various studies as harmless commensals in those that are immune competent.

Entamoeba dispar is a harmless commensal present in the immune competent host and has not been implicated in causing disease or invading the mucosa of patients with AIDS (Stanley Jr., 2003).

Microsporidia sp, such as *Enterocytozoon bienersi* are categorised as definite pathogens since they have been reported to cause chronic diarrhoea in HIV infected individuals and self-limiting acute diarrhoea, in immune competent individuals (Chawla and Ichhpujani, 2011). In recent years, *Isospora belli* has emerged as an important gastrointestinal pathogen listed in Table 2.1 Infections with this organism is most commonly associated with AIDS patients and is known to cause severe diarrhoea (Mudholkar and Namey, 2010). *Sarcocystis hominis* is listed as a possible pathogen since humans act as the intermediate host. During this phase, the host may experience diarrhoea as a result (Chiodini *et al.*, 2003).

Table 2.1.: Virulence and Host Immune Status Associated with Gastrointestinal

Pathogens

Gastrointestinal protozoa	Virulence	Association with host immune status
<i>Balantidium coli</i>	Definite pathogen	2
<i>Blastocystis hominis</i>	Probable pathogen	2
<i>Chilomastix mesnili</i>	Non-pathogen	1
<i>Cryptosporidium parvum</i>	Definite pathogen	1+2
<i>Cyclospora cayetanensis</i>	Definite pathogen	1+2
<i>Dientamoeba fragilis</i>	Possible pathogen	1+2
<i>Endolimax nana</i>	Non-pathogen	1
<i>Entamoeba coli</i>	Non-pathogen	1
<i>Entamoeba dispar</i>	Non-pathogen	1
<i>Entamoeba hartmanni</i>	Non-pathogen	1
<i>Entamoeba histolytica</i>	Definite pathogen	1+2
<i>Enterocytozoon bienersi</i>	Definite pathogen	1+2
<i>Giardia lamblia</i>	Definite pathogen	1+2
<i>Iodamoeba butschili</i>	Non-pathogen	1
<i>Isospora belli</i>	Definite pathogen	2
<i>Sarcocystis hominis</i>	Possible pathogen	1

1: Immune competent host, 2: Immune compromised host and 1+2: Immune competent and immune compromised host.

Cryptosporidium parvum, *Entamoeba histolytica* and *Giardia lamblia* are classified as definite pathogens, as shown in Table 2.1. These entero-pathogenic protozoa are associated with both sporadic as well as outbreak-related diarrhoea (Tigabu, 2010 and Jarmey-Swan *et al.*, 2001). They are important causes of diarrhoea since they are able to infect the immune competent host as well as the immune suppressed host (Wellington *et al.*, 2008, Leav *et al.*, 2003). This chapter will focus mainly on the history, morphology, pathogenesis, treatment and diagnostic techniques used for these organisms.

2.4.1. *Entamoeba histolytica*

2.4.1.1. History and use as a biological weapon

E. histolytica was initially described in 1875 by Fedor Lösh. However, it was in 1903 that Fritz Schaudinn named the species (Fotedar *et al.*, 2007). *E. histolytica* can be used as a biodefense tool. This is primarily because *E. histolytica* has a low infectious dose of < 10 cysts and its cysts have the ability to withstand the effects of chlorination. These properties can be misused for activities such as bioterrorism and war (Barwick *et al.*, 2002).

2.4.1.2. Morphology

E. histolytica cysts are round with a diameter of 10-15 micrometres. The wall around the cell is refractive and is composed of chitin. The refractive wall of the cyst is visible during screening and may help distinguish cysts from artefacts. The contents of the cyst include, four nuclei, glycogen and chromatid bodies. *E. histolytica* trophozoites are motile and range in size from 10-50 micrometres (Stanley Jr., 2003). These characteristics can be observed in Figure 1.

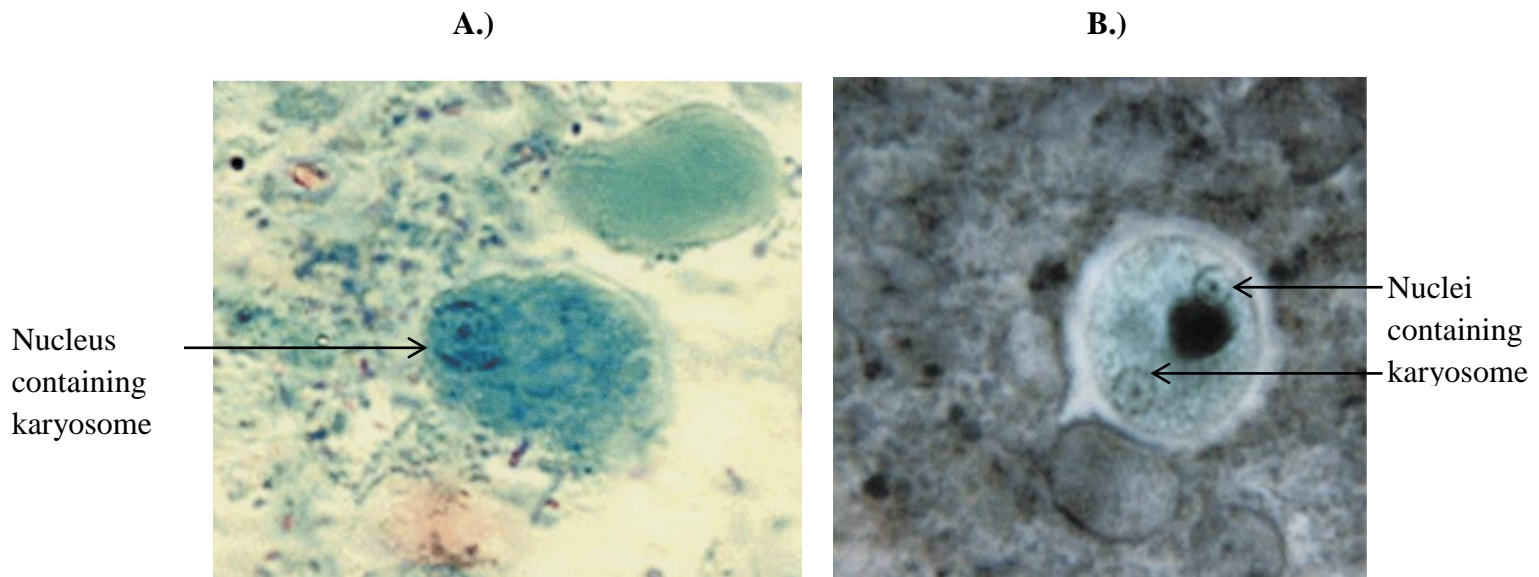


Figure 1: *Entamoeba histolytica* in stool

In the first image (A), *E histolytica* trophozoites are stained with Wheatley's trichrome stain (800 x). The nucleus and central karyosome is visible. The second image (B) shows an *E histolytica* cyst (1600 x) present in stool specimens that were stained with the chlorazol black stain. Two nuclei, each containing the central karyosome, are visible. Figure 1 has been provided by the US Centres for Disease Control and Prevention (Stanley Jr., 2003).

The genus *Entamoeba* consists of two morphologically identical organisms, a commensal, *Entamoeba dispar* and a pathogen, *Entamoeba histolytica* (Fotedar *et al.*, 2007).

Microscopy does not distinguish *Entamoeba dispar* from *Entamoeba histolytica* and is therefore not suitable as a diagnostic method. Methods that can be used include ELISA and PCR (Stanley Jr., 2003).

2.4.1.3. Symptoms and Signs of Amoebiasis

E.histolytica is known to cause amoebiasis (Stanley Jr., 2003). Infection caused by *E. histolytica* can be asymptomatic or symptomatic. In symptomatic patients, this pathogen is known to cause ulcerative, invasive disease of the wall of the colon with bloody diarrhoea (Haque *et al.*, 2003). It can also spread, to the liver causing liver abscess as well to the

respiratory tract, the genitourinary tract and the cerebrum (Fotedar *et al.*, 2007). Children infected with *E. histolytica* are three times more likely to be malnourished and five times more likely to be stunted. Children are therefore regarded as a vulnerable population (Mondal *et al.*, 2006).

2.4.1.4. Pathogenesis and Life Cycle

E. histolytica differs from other protozoa in that it has a relatively simple life cycle, existing either as an infectious cyst or an amoeboid trophozoite. After ingestion, cysts pass through the stomach surviving the hostile acidic environment with its aggressive digestive enzymes. These then travel to the small intestine, where they excyst (Stanley Jr., 2003 and Petri Jr *et al.*, 2002). Eight trophozoites from each cyst are produced.

Adherence of *E. histolytica* to the colonic mucus and intestinal epithelium of the large intestine is mediated by the Gal/GalNAc lectin present on the surface of trophozoites. The Gal/GalNAc lectin binds to carbohydrates of the host that contain Gal and / or GalNac. The Gal/GalNAc lectin has demonstrated the ability to bind to other substrates of the host which include; erythrocytes, neutrophils and colonic mucin glycoproteins. After adhesion, the trophozoites colonize the large intestine (Petri Jr *et al.*, 2002).

E. histolytica kills targeted host cells by adhering to them, inducing their death and then ingesting them. Lectin mediated adherence is initiated by the parasite, thereafter the intracellular calcium is increased and host proteins are dephosphorylated. The parasite completes the kill process by tricking the host cell to induce apoptosis, resulting in host cell

death (Ralston and Petri Jr, 2011). Figure 2 illustrates the cytotoxic effect of *E. histolytica* on the host.

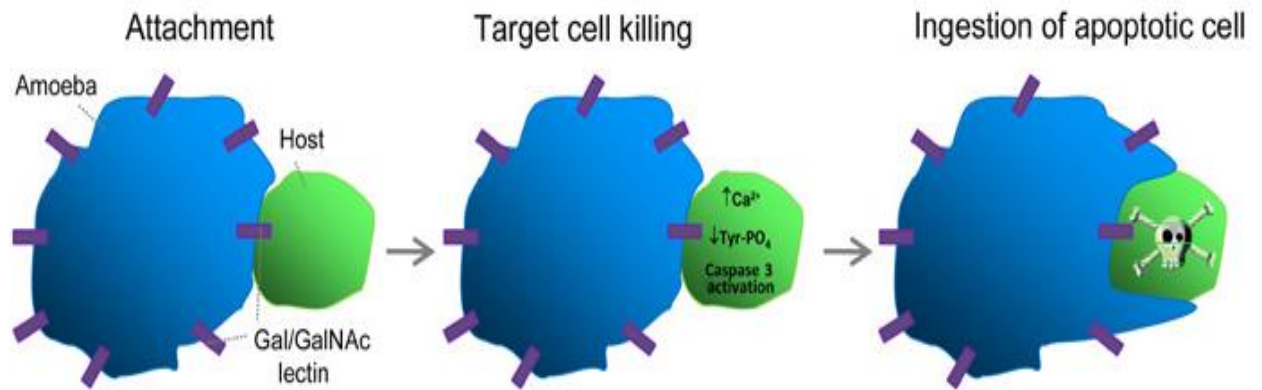


Figure 2: Schematic Diagram of Stepwise Killing and Ingestion of Host Cells by *E. histolytica* (Ralston and Petri Jr, 2011)

In the first step, *E. histolytica* attaches to the host cell by establishing a Gal/GalNAc lectin bond on the outer surface of the cell. Step two demonstrates the biochemical processes that occur and lead to host cell induced apoptosis. In the last step *E. histolytica* ingests the host cell.

Bacteria and food particles are also ingested by the trophozoite. Reproduction occurs in the colon by a process known as binary fission, encystation then follows. The infectious cysts are shed into the environment via the faeces (Stanley Jr., 2003). The life cycle of *Entamoeba histolytica* is shown in Figure 3.

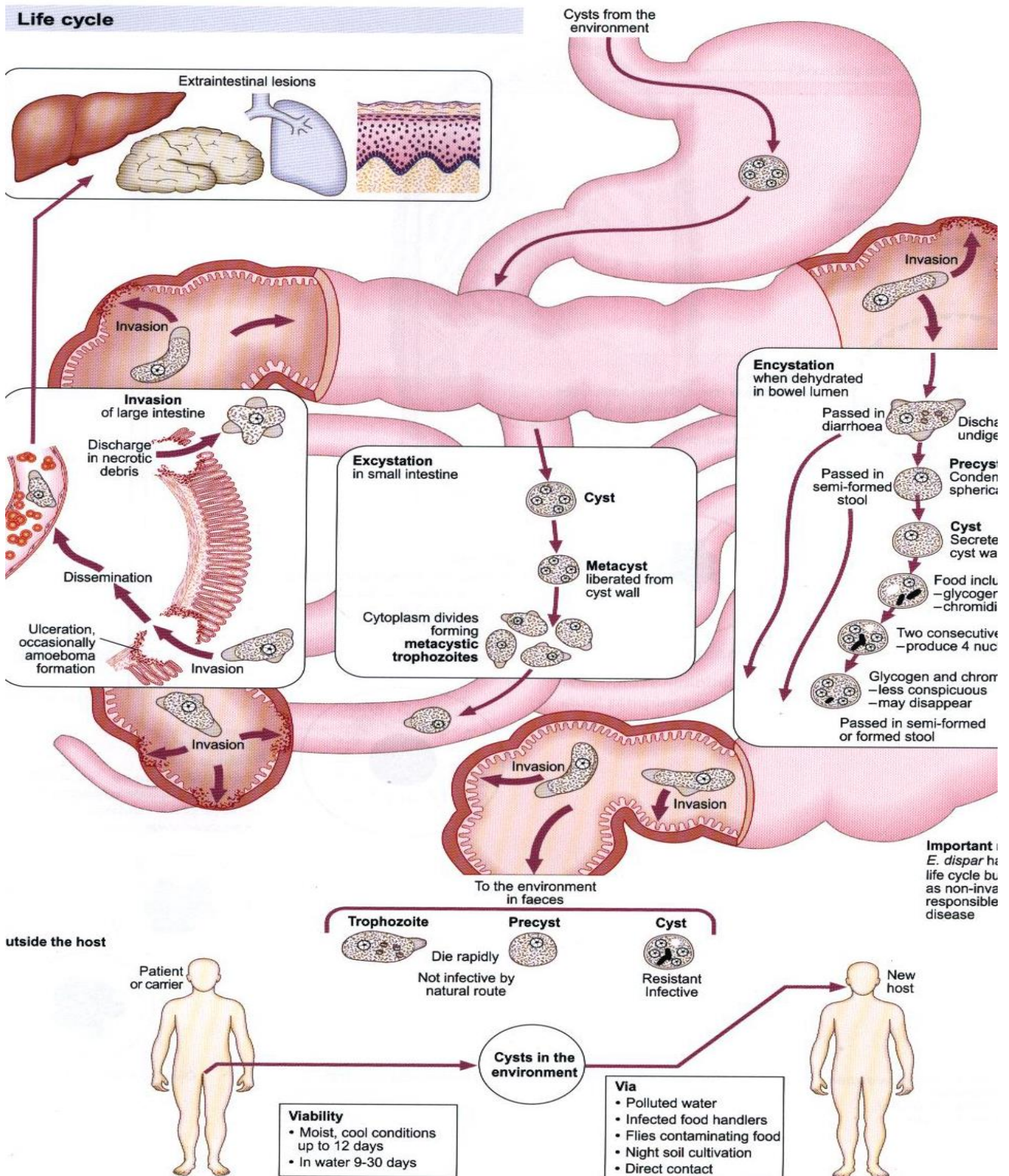


Figure 3: The Life Cycle of *E. histolytica* (Chiodini *et al.*, 2003)

E. histolytica cysts are ingested from the environment. The cysts pass down to the small intestine where they excyst and divide to form trophozoites. Invasion occurs in the large intestine, however the trophozoites may also disseminate to areas such as the liver and brain. When the lumen of the bowel is dehydrated, encystation occurs. Infectious cysts are passed to the environment with the faeces.

2.4.1.5. Treatment

Nitroimidazoles such as metronidazole, tinidazole and ornidazole are used in the treatment of diarrhoea caused by *E. histolytica* (Stanley Jr., 2003).

2.4.1.6. Diagnostic Methods used in the Detection of *E. histolytica*

Microscopic techniques used include, wet-preps, concentration methods, the trichrome stain and the iron-hematoxylin stain. Wet preparations, are performed on fresh stool usually within one hour of collection and has a sensitivity of <10%. Concentration methods are helpful in the demonstration of cysts in asymptomatic patients; however, staining methodologies are helpful in the recovery and identification of *Entamoeba* species (Fotedar *et al.*, 2007).

Culture media used include xenic and axenic systems, with xenic systems referring to growth in the presence of an undefined flora and axenic systems referring to growth without other metabolizing cells being present. Xenic media that can be used include monophasic TYSGM-9, Robinson's medium and diphasic Locke-egg, whilst axenic media used include; TYI-S-33 and YI-S (Fotedar *et al.*, 2007).

Serological tests are also used, however their usefulness is limited to areas in which *E. histolytica* infection is uncommon. In endemic areas the problem that arises is differentiating between antibodies from past and present infections. Another disadvantage is that preserved stool specimens contain preservatives that may denature the target

antigens. In addition, a thousand trophozoites need to be present per well for successful detection (Fotedar *et al.*, 2007).

DNA based diagnostic tests such as PCR, is limited to research laboratories and developed countries. Due to the lack of facilities to conduct DNA based tests as well as the cost incurred, less sensitive detection methods are used in developing countries. Reports suggest PCR of the 18S rDNA gene is a hundred times more sensitive than the best ELISA kit available (Fotedar *et al.*, 2007).

2.4.2. Giardia lamblia

2.4.2.1. History

Giardia was initially described in 1681 after Leeuwenhoek reported on the examination of his own diarrheal stool (Dobell, 1920). The organism was described further by Lambl in 1859. Lambl named it *Cercomonas intestinalis*, as he had thought it belonged to the genus *Cercomonas* based on the morphological resemblances (Lambl, 1859).

There was much controversy emanating from naming this organism, since some scientists named the organism according to the observed morphology, whilst others named the organism based on the host in which it originated from. In 1888, Blanchard suggested the name *Lamblia intestinalis*, (Blanchard, 1888).

In 1902 Stiles, suggested the name to *Giardia duodenalis* (Stiles, 1902). Shortly thereafter, Kofoid and Christiansen suggested the names *G. lamblia* in 1915 and in 1920, the name *G. enterica* (Kofoid and Christensen, 1915 and 1920). In the 1970s the name *G. lamblia* was widely accepted and used (Adam, 2001).

2.4.2.2. Morphology

G. lamblia trophozoites are pear shaped with a diameter of 5-9 micrometres and a length of 12-15 micrometres. The cytoskeleton is made up of a median body and a ventral disk. Two nuclei are present and are identical in appearance. In addition these nuclei replicate at the same time. Four pairs of flagella also constitute the morphology. The trophozoites also

have an axostyle which is composed of two axonemes that can be seen in Figure 4. During microscopic observations the axostyle, nuclei, parabasal (median) body and flagella may be visible. *G. lamblia* cysts measure 5 by 7-10 micrometres in diameter. The cysts are composed of an outer filamentous layer and a double inner membranous layer (Adam, 2001).

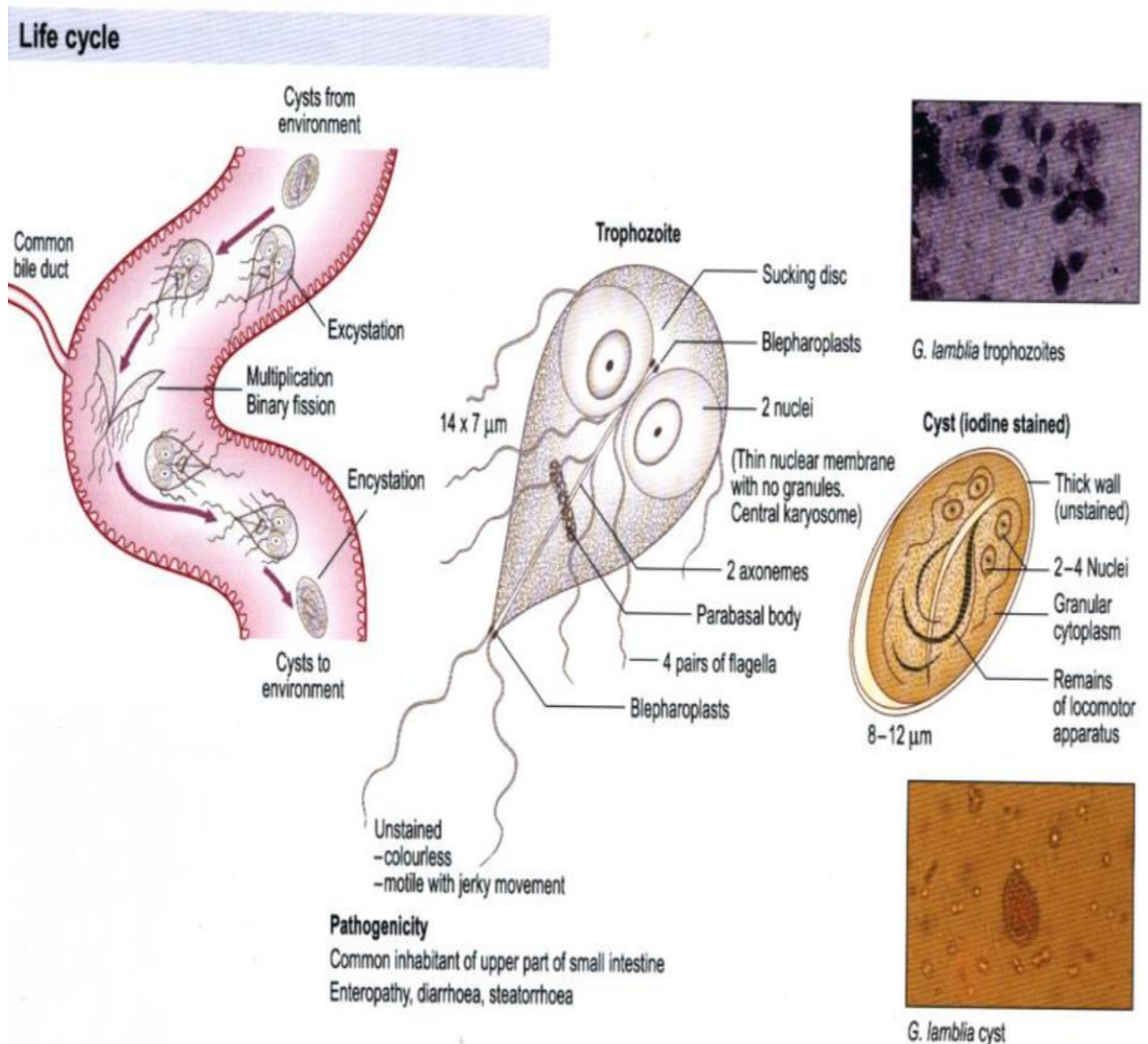


Figure 4: *G. lamblia* Morphology and Life Cycle (Chiodini *et al.*, 2003)

G. lamblia cysts are ingested from the environment and pass to the small intestine, where they excyst to form trophozoites. After excystation the trophozoites undergo binary fission in the small intestine. In the jejunum the trophozoites encyst and are then shed into the environment with the faeces.

2.4.2.3. Transmission

Infectious *G. lamblia* cysts are transmitted through faecal contaminated water and food or through person to person contact. *G. lamblia* has a low infectious dose, requiring only 10 cysts to establish infection (Rendtorff, 1954). This cyst is resistant to chlorination and can survive for a week in cold water (deRegnier *et al.*, 1989 and Johns *et al.*, 1995).

2.4.2.4. Symptoms and Signs

One to three weeks after cysts are ingested, clinical symptoms such as diarrhoea, flatulence, malaise, abdominal cramps and the presence of greasy stools can be observed (Wolfe, 1979 and Hill, 1993). Patients may also display fever, bloody or mucoid stool, vomiting, bloating and anorexia (Moore *et al.*, 1969).

2.4.2.5. Life Cycle

Two major stages occur in the life cycle of *Giardia lamblia*. Ingested cysts pass through the stomach, withstand the low pH and digestive enzyme activity and travel to the small intestine where they excyst into trophozoites. Trophozoites replicate in the small intestine whilst simultaneously causing diarrhoea and malabsorption. In the jejunum the trophozoites encyst and are excreted with the faeces. Upon release into the environment a new host awaits the infectious cyst (Adam, 2001). The life cycle is shown in the Figure 4.

2.4.2.6. Detection Methods

Various detection methods exist for identification of cysts and trophozoites. The concentration of giardia cysts vary in the stool over time which is a diagnostic challenge. Culture techniques are also used but these are limited to research centres. Serological techniques are generally used in epidemiological surveys (Gardener and Hill, 2001). The trichrome stain was reported by Idris and Al-Jabri (2001) to be superior to all other microscopic based identification techniques investigated. This is because it has the ability to detect both cysts and trophozoites of protozoa. Ultimately PCR-based methods have been shown to be more sensitive as well as specific when compared to microscopy and antigen detection tests (Verweij *et al.*, 2004).

2.4.2.7. Treatment

G. lamblia is treated with nitroimidazoles such as, metronidazole, secnidazole, ornidazole and tinidazole. However, the challenge with using nitroimidazoles is the inability of the drug to penetrate the cyst wall. Hence effective removal of the trophozoites is questionable. Gardener and Hill (2001), reported *in vitro* resistance of *G. lamblia* to metronidazole.

2.4.3. *Cryptosporidium parvum*

2.4.3.1. History

In 1907, Ernest Edward Tyzzer first described *Cryptosporidium* species. However, it was only recognised as a pathogen in 1955, during an outbreak of diarrhoea among a flock of turkey. The first case of *Cryptosporidium* infection in humans was reported in 1976. In later years, *C. parvum* was recognized as an important cause of diarrhoea with immune impaired symptoms as a result of the emergence of AIDS. It was only in 1987 that it was recognised as the cause of waterborne infections in immune-competent individuals (Rose, 1988).

2.4.3.2. Morphology

Of primary importance, is the oocyst stage, as dispersal, survival and infection occur during this phase. In addition it is also the stage in which detection as well as identification of the parasite is made (Fayer *et al.*, 2000). Identification of *C. parvum* is challenging as oocysts can easily be confused with yeast cells, algae and plant debris present in cells. Differentiation of species is challenged by the fact that most oocysts are 4-6 µm, nearly spherical and possess obscure internal structures (Fayer *et al.*, 2000).

The oocyst wall of *C. parvum* is made up of rich disulphide bonds (Mitschler *et al.*, 1994), which is protective to the infectious sporozoites. The oocyst walls have two layers, an inner and an outer layer. The outer layer is composed of acidic glycoproteins (Reduker *et al.*, 1985), whilst the inner layer is filamentous and composed of glycoproteins. The central

glycolipid / lipoprotein layer is believed to be responsible for the rigidity and elasticity of the wall (Bonnin *et al.*, 1991).

2.4.3.3. Life Cycle

The life cycle of *C. parvum* includes a sexual and an asexual phase. Once the infectious form is ingested, changes in the environmental conditions of the host such as temperature, pH, pancreatic enzymes and bile salts, result in excystation of four sporozoites from each oocyst (Reduker *et al.*, 1985). These sporozoites adhere to the luminal surfaces of epithelial cells of the infected tract. Microvilli of epithelial cells surrounding the sporozoite facilitate intracellular uptake of the sporozoites. After microgametes and merozoites leave the host cell, they go on to infect other cells. In the parasitophorous vacuole, the endogenous life cycle occurs (Tzipori and Widmer, 2000). Development of the parasite is facilitated by the protective effect exhibited by the membrane of the parasitophorous vacuole, against the host defence system.

The presence of a feeder organelle between the cytoplasm of the host and the parasite, allows for energy and nutrient uptake from the host (Carey *et al.*, 2004). After two generations of successful merogony, Type 1 and Type 2 meronts that contain 6-8 and 4 merozoites, respectively, are released. When a mature merozoite infects another host cell, the merozoite develops into a Type 1 or Type 2 meront, this is known as asexual multiplication.

Type 2 meronts differentiate into microgamonts and macrogamonts, the sexual component of the life cycle. Microgamonts become multinucleated, with each nucleus being incorporated into a microgamete. When the microgamete is fertilized, the macrogamont undergoes division twice to become an oocyst. Thin-walled as well as thick-walled oocysts develop during sporogony, with each oocyst containing four sporozoites that have the potential to cause infection.

Autoinfections as well as persistent infections are the result of thin-walled oocysts that remain within the host. Oocysts that possess a thick wall are shed into the environment via excretion of faeces. The life cycle of *C. parvum* is quite rapid occurring within twelve to fourteen hours of ingestion. Therefore few oocysts are required to cause infection (Current and Garcia et al., 1991). The life cycle of *C. parvum* is shown in Figure 5.

Life cycle

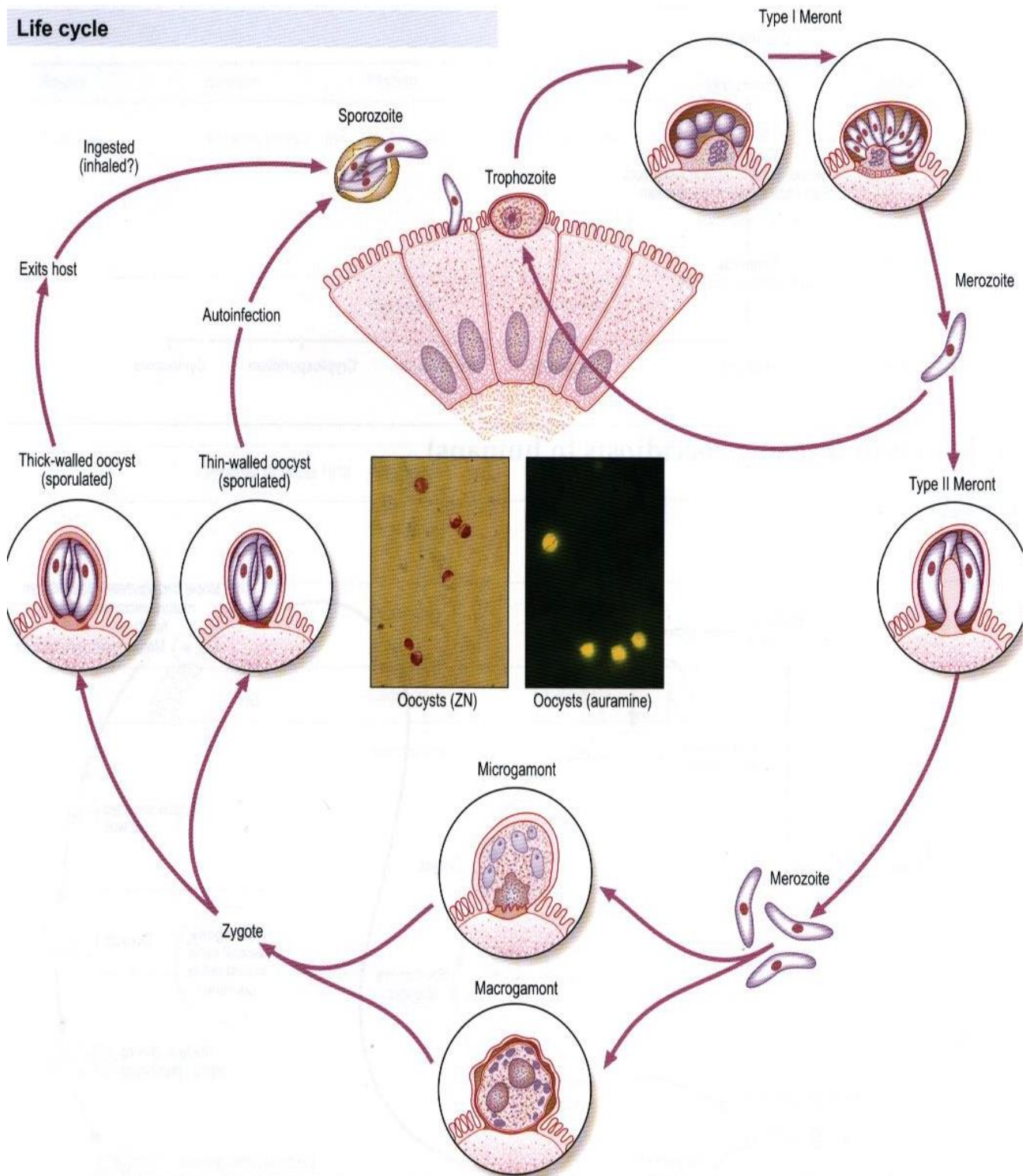


Figure 5: *C. parvum* Life Cycle (Chiodini *et al.*, 2003)

C. parvum undergoes two phases; a sexual phase and an asexual phase. Once oocysts are ingested, they excyst to form sporozoites. With asexual replication a mature merozoite develops into a Type 1 or Type 2 meront. With sexual replication, a Type 2 meront divides to form a microgamont and macrogamont. Sporogony results in the formation of thick-walled oocysts and thin-walled oocysts. Thick-walled oocysts are excreted with the faeces and are released into the environment.

2.4.3.4. Survival and Transmission

Oocyst survival is largely dependent on temperature, as oocysts have the ability to survive in temperatures between 4°C-22°C (Pokorny *et al.*, 2002). Furthermore, cysts can survive in sea water as well as fresh water. Although an increase in temperature speeds up the process of oocyst degradation, oocysts are still known to be infective for twelve weeks at 25 °C in water (Fayer *et al.*, 1996).

Transmission of oocysts via the faecal-oral route is mediated by numerous factors. These factors include person to person contact either through direct contact or indirect contact. Animals may also transmit oocysts to other animals or to humans. In cases of zoonotic transmission, infected humans such as farmers generally contract the infection due to their close contact with animals. Oocysts may be present in recreational water that is used for activities such as swimming and water that is used for consumption. When oocysts are transmitted through food, it is generally due to contamination emanating from lack of hygiene of food handlers.

Air borne transmission occurs through dispersal of oocysts present in soil by environmental conditions such as wind. Faecal contamination of the soil and surface water with *C. parvum* oocysts may lead to further contamination of fresh fruits and vegetables, drinking water and recreational water. Sexual activities have also been documented in the transmission of oocysts (DuPont *et al.*, 1995). However, spread through the faecal-oral route with water as the main vehicle of transmission is a global concern and threatens drinking water safety worldwide (Carey *et al.*, 2004).

Conventional methods to eradicate *C. parvum* from water supplies, such as coagulation, flocculation, sedimentation, filtration and chemical treatment, have proven to be unsuccessful (Carey et al., 2004). Chlorine is effective in killing bacteria but not oocysts. Hence coliform counts which are generally used as indicators for faecal contamination, do not provide evidence for the presence or absence of *C. parvum* (Chauret et al., 1995). No biological indicators exist to determine the presence of *C. parvum* (Carey et al., 2004).

Due to the small size of oocysts, they can pass through most conventional filters. Removal of oocysts is possible with one micrometer absolute filters and reverse-osmosis filters (Steiner et al., 1997). For effective removal of oocysts chemical and physical treatment should be incorporated (Carey et al., 2004).

2.4.3.5. Detection

Various detection methods exist for *C. parvum*. These include concentration and staining of faecal smears as well as differential staining techniques listed in Table 2.2.

Table 2.2.: Differential Staining Techniques for *C. parvum*

Staining technique	Colour of the visible oocysts	Colour of background stain
Ziehl-Neelsen stain	Red	Blue or green
Kinyoun stain	Pale or bright pink	Dark green or blue
Safarin-methylene blue stain	Orange to red	Blue
Auromine-phenol fluorochrome stain	Yellow/green/orange	Red
Carbol fuchsin stain	Bright red	Light blue
DMSO- stain	Red	Green
Nigrosin stain	Unstained halos	Pink
Malachite green negative stain	Unstained halos	Green

Various staining techniques can be used to differentiate *C. parvum* from other organisms present. Oocysts either stain a different colour compared to the background or may possess a halo around the oocyst wall.

Negative staining methods, stain bacteria, yeasts and the background, but not oocysts. This requires examination by an experienced microscopist (Fayer *et al.*, 2000). The Ziehl-Nelsen, Kinyoun and carbol fuchsin stains either stain the background green or blue since it depends on which counter stain is available for use. Generally it is either malachite green or methylene blue.

PCR is also used as an alternative to conventional diagnosis. Immunological-based detection methods such as, ELISAs, immunofluorescence, reverse passive haemagglutination and immunochromatographic assays, among others may also be used (Fayer *et al.*, 2000). Within the *Cryptosporidium* genus, antigens of the oocyst wall are conserved hence monoclonal antibody testing is not advised, as it does not differentiate between the different species of *Cryptosporidium* (Fayer *et al.*, 2000).

2.4.3.6. Treatment

C. parvum can be treated with nitazoxanide, which is a derivative of nitrothiazolyl-salicylamide (Rossignol and Stachulski, 1999). This has been demonstrated in both cell culture and animal models (Theodos *et al.*, 1998, Blagburn *et al.*, 1998 and Gargala *et al.*, 2000). A three day course of nitazoxanide is successful in treating intestinal protozoal infections (Abaza *et al.*, 1998, Romero *et al.*, 1997 and Vásquez *et al.*, 1998). Nitazoxanide is effective against *C. parvum* in AIDS patients. However, the efficacy varies based on the duration of treatment and the degree of immunosuppression (Davis *et al.*, 1996 and Rossignol *et al.*, 1998).

Chapter Three: Materials and Method

3.1. Study Design

This study is a cross-sectional study, which aimed to determine the prevalence of protozoal parasites causing diarrhoea in children aged 0-5 years in the eThekweni district of KwaZulu-Natal.

The study population consisted of children in that age group, who attended the King Edward VIII hospital's outpatient clinic or were admitted to the gastroenteritis ward. Children, producing loose stools frequently were enrolled in the study. King Edward VIII Hospital was chosen as the study location, since it is within close proximity of the university's medical microbiology laboratories.

The sample size was calculated by taking an estimated prevalence of 29%, $p = 0.29$ (Mukhopadhyay et al., 2007, Adamu et al., 2006, Tigabu et al., 2010, and Nxasana et al., 2014). A precision of $\pm 8\%$ with a confidence of 95% was accepted, which resulted in a minimum sample size of 124 participants

3.2. Ethical Considerations

Ethical approval to conduct this study was obtained from the University of KwaZulu-Natal's Biomedical Research Ethics Committee (BREC). The ethics number for this study was BE222/13.

3.3. Specimen Collection

Prior to the collection of stool, the research nurse and I had given the caregivers a detailed explanation of the purpose of the study and the process of informed consent.

Questionnaires were also conducted and caregivers were encouraged to co-operate, as part of the enrolment process. Stool specimens from 249 children were collected in sterile leak-free universal containers. These containers were labelled with the participants' study numbers. Stool specimens arrived at the laboratory on the same day of collection. On arrival at the laboratory, part of the specimen was stored at -20°C for molecular analysis and the remaining stool was preserved in phosphate buffered formalin (5% v/v). The preserved specimens were stored in the cold room at a temperature of 2-8°C.

3.4. Microscopic Analysis

Detection of protozoal cysts in stool was done by means of light microscopy. Preserved specimens were smeared onto glass microscope slides. The smears were allowed to dry which was followed by a staining technique suitable for the protozoan we were attempting to detect.

Two staining techniques were applied: the modified trichrome stain which allows detection of most species of protozoa and the modified Ziehl-Neelsen stain which is a differential staining technique for the acid fast oocysts of *Cryptosporidium parvum*.

3.4.1. The modified Trichrome Stain According to the Ryan-Blue Protocol

The modified trichrome stain was performed according to the Ryan-Blue protocol. The components to make up the staining solution are shown in Table 3.1. The solution was prepared by adding 3 ml of acetic acid to the dry ingredients. The container with the solution was covered with aluminium foil, incubated for 30 minutes at room temperature in the dark. Following this, 400 ml of distilled water was added and the pH was adjusted by adding drops of 1.0 M HCl till the required pH of 2.5 was reached.

Table 3.1.: Formula for the Modified Trichrome staining solution

Chemical Components	Quantity	Concentration
Chromotrope 2R	24 g	0.13M
Aniline blue	2 g	0.006M
Phosphotungstic acid	1 g	0.9M
Acetic acid (glacial)	12 ml	0.5M
Distilled water	400 ml	

The stored specimen was mixed till an even suspension was obtained. Using a Pasteur pipet, a drop was placed onto a microscopy slide. This was air-dried at 37°C. Thereafter slides were placed in the trichrome stain for 90 minutes. This was followed by rinsing with 3 % acid alcohol. To remove the acid, slides were dipped seven times in 95% ethanol. This was followed by placing the slide in 100% ethanol for 3 minutes. This last step was repeated before exposure to accustain exylene for 10 minutes. Slides were then air dried at room temperature and a cover slip was mounted on top of the specimen using DPX (Ryan *et al.*, 1993).

3.4.2. The Modified Ziehl-Neelsen Staining Technique

For the modified Ziehl-Neelsen technique, a drop of the faecal specimen was placed onto a microscopy slide and air dried at 37°C. The material was stained with carbol fuchsin for 20 minutes, rinsed under the tap and decolourised with 3% acid alcohol for 20 seconds. The slide was rinsed under the tap and counter stained with methylene blue for 60 seconds. A final rinse was performed under the tap and the slides were left to air dry. Once dry, a cover slip was mounted on top of a smear using DPX (Adamu et al., 2006).

3.4.3. Microscopy

An Olympus BX40F-3 model bright field microscope was used to screen specimens at 400 x magnification. Suspected objects were then viewed at 1000 x magnification. The entire area (22mm x 7mm) under the cover slip was screened. Images of the cysts and oocysts were captured using the Image Pro-Plus camera software on the Leica DM 3000 microscope.

3.4.4. The Formol-Ether Concentration Technique

A pea-size portion of stool was added to an unused stool container and emulsified in 10 ml of 4% formaldehyde in saline. The suspension was then filtered through gauze into a clean 20 ml conical collection tube and 3 ml of ether was added to the filtrate. The tube was closed and shaken vigorously for 30 seconds.

Thereafter the suspension was filtered once again through gauze into a clean 20 ml conical collection tube. After centrifugation four layers had formed viz. the ether layer, the plug of debris, the layer containing the 10% formal saline and the last layer containing the deposit of intestinal parasites. The ether, plug of debris and formal saline layers were decanted into a waste bucket and the deposit was kept in the fridge at 4°C.

3.5. DNA Extraction Techniques

Different DNA extraction methodologies were explored to obtain DNA from *C. parvum* oocysts and *G. lamblia* cysts. Four clinical specimens that tested positive on microscopic examination were used to test DNA extraction methods. In addition, four positive specimens (*C. parvum* 1, *C. parvum* 2, *G. lamblia* 1 and *G. lamblia* 2) obtained from a private laboratory were also used. These were specimens that were not preserved in formalin. To confirm the presence of *C. parvum*, Ziehl-Neelsen stained smears were prepared and viewed. Similarly, iodine stained wet-preps were made with *G. lamblia* positive specimens. The different DNA extraction methods tested are listed in Table 3.2.

DNA was quantified and qualitatively assessed with the Thermo Scientific Nanodrop 2000c spectrophotometer. DNA was considered “good” if the quantity was >10 ng/μl. The quality of the DNA was deemed satisfactory if the 260/280 ratio was between 1.8-2.

Table 3.2.: DNA Extraction Techniques

DNA Extraction Techniques	
A. The QIAamp Fast DNA Stool Mini Kit	Qiagen Hilden, Germany
B. CTAB Method	Van Embden <i>et al.</i> , 1993
C. QIAamp DNA Mini Kit (protocol for blood specimens)	Qiagen Hilden, Germany
D. QIA amp DNA Stool Mini Kit (protocol for stool specimens)	Qiagen Hilden, Germany

3.5.1. QIAamp Fast DNA Stool Mini Kit Protocol (A)

The kit manufacturer's instructions were followed. All reagents and buffers were provided with the kit.

A pea-size amount of stool was added to a clean 2 ml microcentrifuge tube and 1 ml of inhibitEx was added, followed by vortex agitation for 1 minute. The suspension was heated at 95°C for 5 minutes to allow lysis of the cysts. After vortexing for 15 seconds, the specimens were centrifuged for 1 minute to remove solid stool particles. Fifteen µl of proteinase K was mixed with 200 µl of supernatant from the previous step and 200 µl of buffer AL. This mixture was vortexed for 15 seconds and incubated for 10 minutes at 70°C.

After incubation, 200 µl of absolute ethanol was added, followed by vortex agitation for 15 seconds. A QIAamp spin column was then placed in a 2 ml collection tube, 600 µl of lysate from the previous step was added to the spin column and this mixture was centrifuged for 1 minute at 14000 x g. After centrifugation, the filtrate from the spin column was discarded and the spin column was transferred to a new 2 ml collection tube. The next step entailed adding 500 µl of wash buffer AW1 to the spin column and centrifugation for 1 minute.

The filtrate from the spin column was discarded and the spin column was placed in a new 2 ml collection tube, followed by the addition of 500 µl of wash buffer AW2 and centrifugation for 3 minutes at 14000 x g. After centrifugation the filtrate from the spin column was discarded and the spin column was placed in a new 2ml collection tube and centrifuged for 3 minutes.

The QIAamp spin column was then placed in 1.5 ml microcentrifuge tube and 200 µl of ATE buffer was added directly onto the membrane of the spin column. A minute of incubation at room temperature and a subsequent centrifugation for 1 minute to elute the DNA then followed.

No measurable amount of DNA was obtained applying this method. Hence a number of different modifications were tried:

1. DNA was eluted in 60 µl of ATE buffer instead of 200 µl of ATE buffer
2. The lysis temperature was increased to 100°C for 10 minutes instead of 95°C for 5 minutes and the DNA was eluted in 60 µl of ATE buffer, instead of 200 µl of ATE buffer
3. An additional step was incorporated prior to performing DNA extraction. This step included adding 180 µl of ATL buffer and 20 µl of proteinase K to the stool

specimens and placing this in a 56°C water bath for 3 hours. The addition of proteinase K in other steps was omitted.

4. Modification number 1 was modified by adding silica beads to a 2ml microcentrifuge tube containing a pea-size portion of the stool. The stool specimen was subjected to three cycles of vortex agitation for 60 seconds and placing on ice for 2 minutes. Centrifugation for 10 minutes at 12000 x g then followed.

3.5.2. Cetyl Trimethyl Ammonium Bromide (CTAB) Method for DNA Extraction (B)

Seventy µl of 10% (w/v) sodium-dodecyl sulphate (SDS) and 50 µl of proteinase K were added to 200 µl of stool. The tubes were placed in a 60°C water bath for 60 minutes followed by the addition of 100 µl of 5M NaCl. The tubes were inverted seven times to mix the suspension. Thereafter 100 µl of warm CTAB was added. The contents of the tubes was mixed by hand inversion and incubated at 60°C for 15 minutes followed by freezing by placing the tubes at -70°C for 15 minutes. This was followed by thawing in a 60°C water bath and 700 µl of chloroform/isoamyl alcohol (24:1) was added and mixed by inversion. The mixture was then centrifuged for 10 minutes at 13000 x g. The upper aqueous layer that formed was then transferred to a new tube. Thereafter, 700 µl of cold isopropanol was added and the tube was inverted seven times.

The extracts were then kept at 4°C overnight followed by centrifugation for 10 minutes at 13000 x g. The fluid was removed and the deposit suspended in 70% (v/v) ethanol. This suspension was centrifuged for 10 minutes at 13000 x g. Ethanol was removed and the remaining DNA was left to air-dry. Based on the size of the resulting pellet, 10-30 µl of 1x

(v/v) TE buffer was used to re-suspend the DNA. The DNA was stored at -20°C till further use.

The DNA obtained with the CTAB method varied in quantity and quality. When the PCRs (3.6) were tried on the good extracts; no amplicons was found. Therefore the formol-ether concentration technique was applied and the CTAB DNA extraction method repeated. This did not improve the results.

3.5.3. QIAamp DNA Mini Kit Protocol for Blood Specimens (C)

The kit manufacturer's instructions were followed. All reagents and buffers were provided with the kit.

A pea-size portion of stool was placed in a 2 ml microcentrifuge tube, 200 µl of TE buffer was added and the suspension was boiled for 15 minutes at a 100°C. The suspension was kept at 4°C overnight and centrifuged for 5 seconds at 8000 x g. The supernatant was aspirated and decanted into a 2 ml microcentrifuge tube. Thereafter, 200 µl of buffer AL and 20 µl of proteinase K were added to the supernatant, followed by vortex agitation for 15 seconds and centrifugation at 8000 x g for 5 seconds. This mixture was then placed in a 56°C water bath for 10 minutes and 200 µl of 70% (v/v) ethanol was added. Vortex agitation for 15 seconds and centrifugation at 8000 x g for 5 seconds, followed.

The lysate from the previous step was added to a spin column and centrifuged for 1 minute at 8000 x g. The spin column was placed in a new 2 ml collection tube and the filtrate and

the collection tube was discarded. Thereafter 500 µl of wash buffer AW1 was added to the spin column and the tube was centrifuged for 1 minute at 8000 x g. The filtrate was discarded, the spin column was placed in a new 2 ml collection tube and 500 µl of wash buffer AW2 was added. Centrifugation for 3 minutes at 14000 x g followed.

The spin column was transferred to a new 2 ml collection tube, the filtrate was discarded and the tubes were centrifuged for 1 minute at 14000 x g. The spin column was placed in a 1.5 ml microcentrifuge tube, the filtrate and collection tube was discarded and 60 µl of elution buffer AE was added to the spin columns. Incubation for 5 minutes at room temperature followed, as well as a subsequent centrifugation at 8000 x g. Thereafter the spin column was discarded and DNA was kept at -20°C.

3.5.4. QIAamp DNA Mini Kit Protocol for Tissue Specimens (D)

The kit manufacturer's instructions were followed. All reagents and buffers were provided with the kit.

A pea-size portion of stool was added to a 2 ml microcentrifuge tube. To this 180 µl of ATL buffer and 20 µl of proteinase K was added. The tubes were vortex agitated for 15 seconds and centrifuged for 5 seconds at 8000 x g. Incubation of this mixture for 3 hours in a 56°C water bath then followed. Two hundred µl of buffer AL was added and the mixture was incubated at 70°C for 10 minutes. Then, 200 µl of 70% (v/v) ethanol was added to the tubes, followed by vortex agitation for 15 seconds and centrifugation at 8000 x g for 5 seconds.

The lysate was added to a spin column and centrifuged for 1 minute at 8000 x g. The spin column was placed in a new 2 ml collection tube and the filtrate was discarded. Five hundred μ l of wash buffer AW1 was added to the spin columns and centrifuged for 1 minute at 8000 x g. The spin column was transferred to a new 2 ml collection tube; the filtrate was discarded and 500 μ l of wash buffer AW2 was added to the spin column.

Centrifugation for 3 minutes at 14000 x g followed. The spin column was then placed in a new 2 ml collection tube, the filtrate was discarded and the tubes were centrifuged for 1 minute at 14 000 x g. The spin column was transferred to a 1.5 ml microcentrifuge tube, the filtrate and collection tube was discarded and 60 μ l of elution buffer AE was added to the spin columns. Incubation for 5 minutes at room temperature and centrifugation at 8000 x g for 1 minute followed. The spin column was discarded and the DNA was kept at -20°C.

To improve the results this DNA extraction technique was repeated following two different procedures applied before the extraction took place:

1. Formol-ether concentration technique of the stool specimens.
2. Vortex agitation (3 x 60 seconds) followed each time by cooling on ice for 2 minutes of the stool specimens with silica beads.

3.6. Polymerase Chain Reaction (PCR)

3.6.1. Initial PCR Protocol

Polymerase Chain Reaction as described by the protocol from the AmpliTaq® Gold DNA Polymerase kit was performed using DNA extracted by the different methods as described in 3.5 from specimens that were confirmed positive for *Cryptosporidium parvum* and *Giardia lamblia*. The concentration and quality of the DNA was determined using a spectrophotometer (Nanodrop), the purity was further confirmed by means of gel electrophoresis.

PCRs were performed according to the AmpliTaq® Gold DNA Polymerase kit's recommendations (AmpliTaq® Gold DNA Polymerase Protocol, Pub. No. MAN0009863 Rev. A.0., 2014, ThermoFisher Scientific) for both *Cryptosporidium parvum* and *Giardia lamblia*. Both PCRs was performed using a 25 µl reaction volume. PCR master mix components are listed in Table 3.3. The amount of template DNA used can be seen in Table 3.4. Apart from the primers (Table 3.5.), the components were the same. Following the kit's recommendations, no amplification of the expected products was achieved. Hence various optimisation steps were implemented.

Table 3.3.: Mastermix Components

Component	Quantity (µl)
10 x PCR buffer II	2.5
10mM dNTP mix	0.5
25mM MgCl ₂	1.5
10µM Forward primer	0.5
10 µM Reverse primer	0.5
Amplitaq gold DNA polymerase	0.125 (0,625 U)
Nuclease-free water	11.375

These components constituted the 25 µl mastermix reaction for *C. parvum* and *G. lamblia*e

Table 3.4. The amount of template DNA used for test specimens

Template DNA	Quantity (ng/8µl)
Specimen 3 (Protocol C)	165.6
Specimen 3 (Protocol D)	556,8
Specimen 183 (Protocol D)	92,8
Specimen 191 (Protocol D)	552
Specimen 294 (Protocol D)	85.6
PL3 (Protocol D)	127.2
PL4 (Protocol D)	136
PL3 (Protocol D: Silica beads)	49.6

DNA extraction protocols yielded sufficient DNA with good purity for 5 specimens: 4 with the tissue protocol (D) and 1 with the CTAB method. PL3 represents a positive *C. parvum* specimen from a private laboratory, and PL4 represents a positive *G. lamblia*e specimen from a private laboratory.

Table 3.5.: *C. parvum* and *G. lamblia*e Primer Sets

Parasite	Primer name	Primer Sequence	Reference
<i>C. parvum</i>	AWA722F (Forward)	5' AGTGCTTAAAGCAGGCAACTG 3'	Elsafi <i>et al.</i> , 2013
	AWA1235R (Reverse)	5' CGTTAACGGAATTAACCAGAC 3'	
<i>G. lamblia</i> e	RH 11 (Forward)	5' CATCCGGTTCGATCCTGCC 3'	Hopkins <i>et al.</i> , 1997
	RH4 (Reverse)	5' AGTCGAACCCTGATTCTCCGCCAGG 3'	

PCRs were performed using the ABI 9700 thermo cycler. Except the annealing temperature, the cycling conditions were the same for both the *C. parvum* and the *G. lamblia*. The cycling conditions are shown in table 3.6. PCR followed a protocol that recommended an initial denaturation at 95°C for 10 minutes to activate the enzyme. A final extension of 72°C for 5 min was performed. The expected product size for *C. parvum* and *G. lamblia* was 556 bp and 292 bp, respectively.

Table 3.6.: PCR Cycling Conditions for *C. parvum* and *G. lamblia*

Conditions	
Denaturation	95°C for 15 seconds
Annealing	52°C (<i>C. parvum</i>) and 59°C (<i>G. lamblia</i>) for 30 seconds
Extension	72°C for 1 minute/kb
No. of cycles	35

The same conditions were used for both parasites. Only the annealing temperature differed. *G. lamblia* displayed a higher annealing temperature than *C. parvum*.

3.6.2. Optimisation of PCRs

The following modifications to the above protocol were tried:

1. 5 µl of DNA was used instead of 8 µl (Table 3.8.). The decreased volume was replaced with additional µl of nuclease-free water.
2. The master mix composition was adjusted as shown in Table 3.7.

Table 3.7.: The Mastermix Components and DNA Template

Component	Quantity (µl)	
	Amplitaq's Protocol	Adjustment
10 x PCR buffer II	2.5	2.5
10mM dNTP mix	0.5	0.75
25mM MgCl ₂	1.5	0.9
10µM Forward primer	0.5	1
10 µM Reverse primer	0.5	1
Amplitaq gold DNA polymerase (5U/µl)	0.125 (0.625 U)	0.2 (1 U)
Nuclease-free water	11.375	13.6 5
Template DNA	8	5

This table represents the modifications made to the AmpliTaq® Gold DNA Polymerase Protocol's mastermix.

Table 3.8. The Initial and Adjusted Amounts of Template DNA Used

Template DNA	Quantity (ng/µl)	
	Initial DNA (8µl)	Adjustment (5 µl)
Specimen 3 (Protocol C)	165.6	103.5
Specimen 3 (Protocol D)	556,8	348
Specimen 183 (Protocol D)	92,8	58
Specimen 191 (Protocol D)	552	345
Specimen 294 (Protocol D)	85.6	53.5
PL3 (Protocol D)	127.2	79.5
PL4 (Protocol D)	136	85
PL3 (Protocol D: Silica beads)	49.6	31

This table shows the reduced amounts of template DNA used

3. The following protocol was tried using the adjusted master mix and template DNA

(Table 3.7. and 3.8.):

- Initial denaturation at 95°C for 5 minutes
- Denaturation at 95°C for 1 minute (35 cycles)

- Annealing at 52°C for 30 seconds
- Extension at 72°C for 1 minute
- Final extension of 72°C for 5 minutes

4. The conditions as advised for the Qiagen Fast cycling PCR kit were applied for the *C. parvum* PCR using the adjusted master mix (Table 3.7.) and template DNA (Table 3.8.):

- Initial denaturation at 95°C for 5 minutes
- Denaturation at 96°C for 5 seconds (35 cycles)
- Annealing at 52°C for 5 seconds
- Extension at 68°C for 17 seconds
- Final extension of 72°C for 1 minute

5. The following conditions for *G. lamblia* detection were tried (Hopkins *et al.*, 1997) using the adjusted master mix:

- Initial denaturation at 96°C for 2 minutes
- Denaturation at 96°C for 20 seconds (35 cycles)
- Annealing at 59°C for 20 seconds
- Extension at 72°C for 30 seconds
- Final extension of 72°C for 7 minutes

6. The annealing temperatures used by Elsafi (2013) and Hopkins (1997) for *C. parvum* and *G. lamblia*e respectively (Table 3.6.), were varied by increments of 1°C above and below the melting temperature (T_m) to a maximum of + 5°C. These temperatures were applied using the adjusted master mix (Table 3.7. and Table 3.8.) and conditions as per Table 3.6.

7. The melting temperature (www.thermofisher.com) was calculated based on the primer sequences shown in Table 3.5 and verified on the Neb T_m calculator. The calculated T_m was 46.4°C for *C. parvum* and 54.3°C for *G. lamblia*e.

3.7. Statistical Analysis

Age in months is the only numeric data. Its frequency distribution did not meet the criteria for normality using Shapiro Wilks test. Therefore the median and interquartile ranges were used as summary statistics and the non-parametric two-sample Wilcoxon rank-sum (Mann-Whitney) test were used to compare positive and negative patients. Chi square tests or Fisher's exact test (depending on the number of observations in each category) were used to compare categorical data such as demographic characteristics of positive and negative patients. A p value of 0.05 was considered statistically significant. Data was analysed using the statistical software Stata V13.1.

Chapter Four: Results

4.1. Microscopy Results

Table 4.1.1 shows the entero-pathogenic protozoa detected by each staining technique.

Two parasites were detected *G. lamblia* (Fig 4.1.1.) and *C. parvum* (Fig 4.1.2.).

E. histolytica was not detected in both staining techniques used. The total number of patients harbouring a parasite is based on observations with both staining techniques. The term NPS refers to “no parasites seen”. There were more *C. parvum* than *G. lamblia* and this difference was significant ($p < 0.001$). *G. lamblia* stained blue with the Ziehl-Neelsen staining technique (Fig 4.1.3.). Two patients were co-infected with both parasites.

Microscopic observations were confirmed by an experienced and independent laboratory technician.

Table 4.1.1.: The Prevalence of Intestinal Parasites Stratified by Staining Technique in Children ≤ 5 years (n=251)

Technique	No. (%)		NPS
	<i>G. lamblia</i>	<i>C. parvum</i>	
Ziehl-Neelsen stain	2 (1)	38 (15.2)	212 (84.5)
Trichrome stain	6 (2.4)	18 (7.2)	227 (90.4)
Total no. of patients	8 (3.2)	52 (20.7)	191 (76)

NPS: Number of parasites seen. Children that tested positive on both stains were counted once to account for the number of total patients infected.

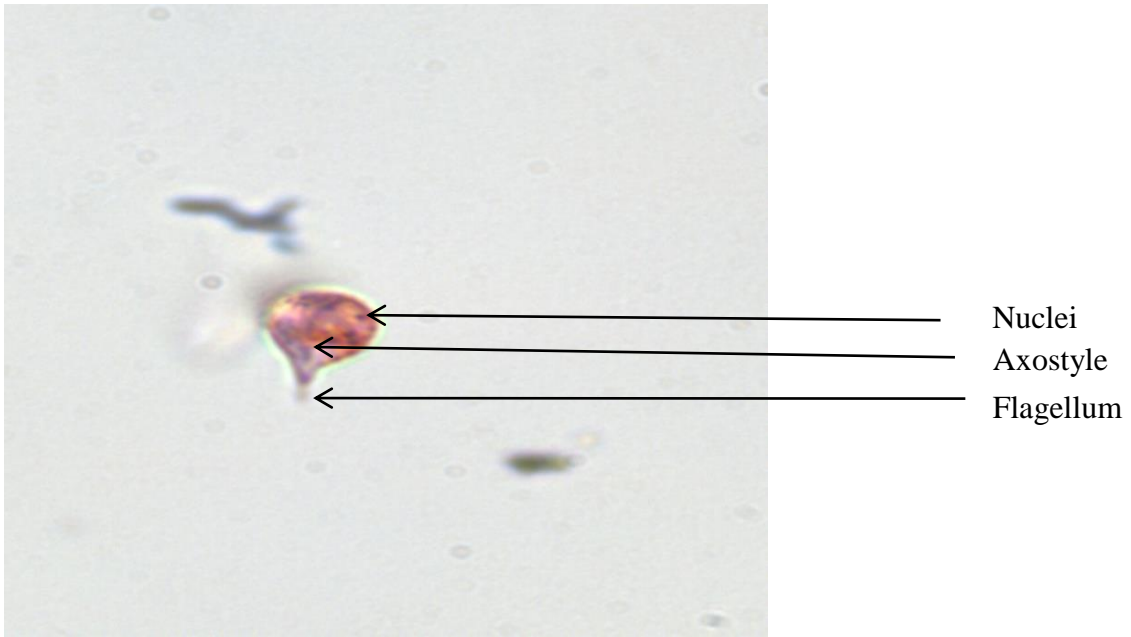


Fig 4.1.1: *G. lamblia* trophozoite x 1000 (modified trichrome stain)

G. lamblia trophozoites stain pink against a light blue background.

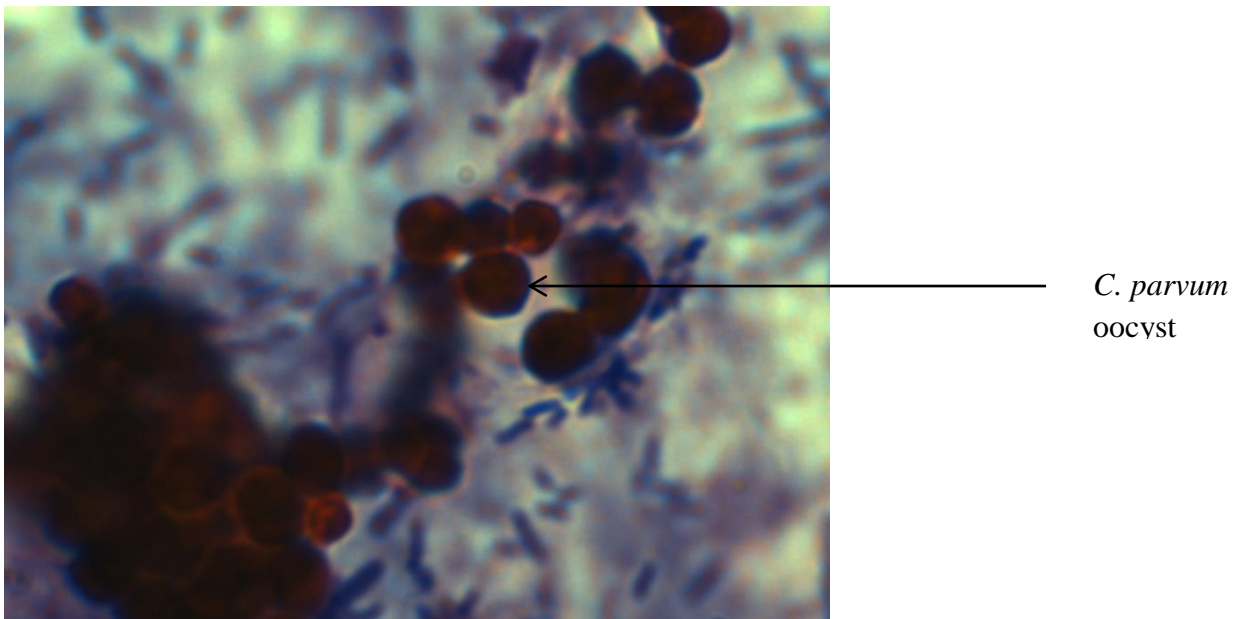


Fig 4.1.2: *C. parvum* oocysts x 1000 (modified Ziehl-Neelsen stain)

C. parvum oocysts stain dark red against a blue background. The cysts displayed a typical round cylindrical shape and were small in size.

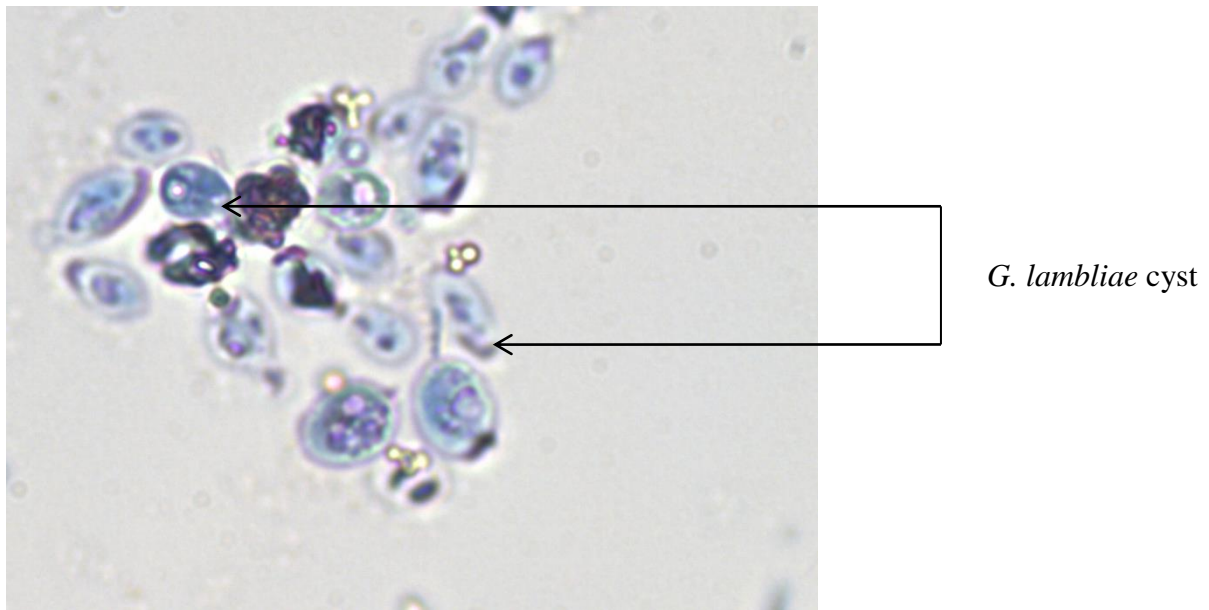


Fig 4.1.3: *G. lamblia* cyst x 1000 (modified Ziehl-Neelsen stain)

Blue stained *G. lamblia* cysts are seen in the background.

The age distribution of the participants is given in table 4.1.2. *G. lamblia* was only found in children ≤ 23 months. *C. parvum* was detected in 42 of the 214 children in that age category. Therefore, the prevalence of both pathogens was, with 88% for *G. lamblia* and 81% for *C. parvum* highest in children ≤ 23 months. The median age for *G. lamblia* positive children was 10 months with an inter-quartile range of 3-18 months. The median age for children harbouring *C. parvum* was 8 months with an inter-quartile range of 4-18 months.

Table 4.1.2.: *G. lamblia* versus *C. parvum* Prevalence Stratified by Age in Children ≤ 5 years (n=251)

Age	No. (%)		Total
	<i>G. lamblia</i>	<i>C. parvum</i>	
< 6 months	2 (25)	17 (33)	76
6-11 months	2 (25)	11 (21)	65
12-18 months	2 (25)	11 (21)	58
19-23 months	1 (12.5)	3 (6)	15
> 24 months	0	7 (13)	29
Unknown	1 (12.5)	3 (6)	8
Total	8 (100%)	52 (100%)	251

The childrens ages were taken from their case charts. Those charts that had missing ages were refered to as “unknown”.

Children belonging to the female gender haboured more protozoal parasites than males, (Table 4.1.3). *C. parvum* was present in 50% of females and 44% of males whilst, *G. lamblia* was present in 50% of female and 40% of male children. The gender was unknown in 3 *C. parvum* positive children and a single *G. lamblia* positive child. These differences were not statistically significant ($p > 0.05$).

Table 4.1.3.: *G. lamblia* versus *C. parvum* prevalence stratified by gender in children ≤ 5 years (n=251)

Gender	No. (%)		Total
	<i>G. lamblia</i>	<i>C. parvum</i>	
Male	3 (37.5)	23 (44)	134
Female	4 (50)	26 (50)	107
Unknown	1 (12.5)	3 (6)	10
Total	8 (100)	52 (100)	251

Table 4.1.4. shows that there was no correlation between an exposed or positive HIV status and children that were positive for *G. lamblia*. The status for 2 individuals that tested positive for *G. lamblia* was unknown. However, of the children infected with *C. parvum* 25% had a history of exposure to HIV and 10% tested positive. Of those that were infected with *C. parvum* 59% were HIV sero-negative and had no history of exposure.

Table 4.1.4.: *G. lamblia* and *C. parvum* prevalence stratified by HIV status in children ≤ 5 years (n=251)

HIV Status	No. (%)		Total
	<i>G. lamblia</i>	<i>C. parvum</i>	
Exposed	0	13 (25)	36
Positive	0	5 (10)	27
Negative	6 (75)	31 (59)	171
Unknown	2 (25)	3 (6)	17
Total	8 (100)	52 (100)	251

There was no known association between *G. lamblia* and HIV. However, an association between HIV and *C. parvum* was observed.

The drinking water source in the households to which the children enrolled in this study belonged, is shown in table 4.1.5. Only one child that was positive for *C. parvum* used water from a water tank, 36% had piped water in their dwelling and 17% used water from a communal tap at the site where they lived. None of the children that had *C. parvum* infection used the river as a water source and in 3 out of 52 *Cryptosporidium* positive children, the water source was unknown. Of the children infected with *G. lamblia*, 25% had piped water in their dwelling and 50% had access to water from a communal tap. One child sourced their water from the river; and the water source used by the household of one other child could not be established. The differences seen in the water source for each parasite were not statistically significant ($p > 0.05$).

Table 4.1.5.: *G. lamblia* and *C. parvum* Prevalence Stratified by Water Source in children \leq 5 years (n=251)

Water Source	No. (%)		Total
	<i>G. lamblia</i>	<i>C. parvum</i>	
Piped in dwelling	2 (25)	19 (36)	75
Piped on site	4 (50)	29 (56)	166
River	1 (12.5)	0	2
Water tank	0	1(2)	1
Unknown	1 (12.5)	3 (6)	7
Total	8 (100)	52 (100)	251

The water source used was mostly piped water; however children still acquired parasitic infections.

Table 4.1.6 summarises the type of sanitation used by children infected with enteropathogenic protozoa. The pit toilet system was used by 19% and flush toilets were used by 73% of *C. parvum* positive children. Only one child that harboured *C. parvum* used the

ventilated improved pit (VIP) latrines toilet system and the sanitation status for 3 children was not known. For *G. lamblia* positive children, 25% used pit toilets and 50% used flush toilets. For one child sanitation status was unknown, the VIP toilet system was used by another child harbouring *G. lamblia*.

4.1.6.: Prevalence of *G. lamblia* and *C. parvum* and toilet system used by households with children ≤ 5 years (n=251)

Toilet System	No. (%)		Total
	<i>G. lamblia</i>	<i>C. parvum</i>	
Flush	4 (50)	38 (73)	195
Pit latrines	2 (25)	10 (19)	39
VIP*	1 (12.5)	1 (12.5)	9
Unknown	1 (12.5)	3 (6)	8
Total	8 (100)	52 (100)	251

The majority of children harbouring either *C. parvum* or *G. lamblia* used a flush toilet system.* Ventilated improved pit (VIP) toilets are pit toilets that have been fitted with a vent pipe and fly screen. This is to reduce odour and flies from the toilet.

The duration of diarrhoea prior to enrolment in the study is shown in table 4.1.7. Of the children positive for *C. parvum*, 58% had diarrhoea lasting between 1 and 3 days, 21% between 4 and 7 days while the remaining 6% had diarrhoea for more than 7 days. The duration of diarrhoea was unknown in 15%. Of the caregivers of the 8 children infected with *G. lamblia* 3 (38%) reported the diarrhoea to be present for 1 to 3 days and 2 (25%) for more than 7 days.

4.1.7.: The Duration of Diarrhoea and Prevalence of *G. lamblia* and *C. parvum* in

Children ≤ 5 years (n=251)

No. of days	No. (%)		Total
	<i>G. lamblia</i>	<i>C. parvum</i>	
1-3 days	3 (37.5)	30 (58)	120
4-7 days	1 (12.5)	11 (21)	95
> 7 days	2 (25)	3 (6)	16
Unknown	2 (25)	8 (15)	20
Total	8 (100)	52 (100)	251

The nutritional status in children carrying an entero-pathogenic protozoon is illustrated in table 4.1.8. Of the children that were infected with *C. parvum* 28 (54%) were malnourished and 4 (14%) showed signs of kwashiorkor. Malnourishment without kwashiorkor was observed in 38% of children infected with *G. lamblia*.

4.1.8.: Nutritional Status in Children ≤ 5 years (n=251) infected with *G. lamblia* and/ or *C. parvum*

Nutritional Status	No. (%)		Total
	<i>G. lamblia</i>	<i>C. parvum</i>	
Normal	4 (50)	21 (40)	110
Malnutrition	3 (37.5)	28 (54)	118
with kwashiorkor	0	4 (14)	15
Unknown	1 (12.5)	3 (6)	8
Total	8 (100)	52 (100)	251

Children were classified as having malnutrition with kwashiorkor based on assessment by the attending clinician.

4.2. DNA extraction results

Before applying DNA extraction and PCR to all clinical specimens, the methodology was tested using microscopically positive specimens. The QIAamp Fast DNA Stool mini kit protocol (protocol A) did not provide DNA of sufficient quantity and quality (Table 4.2.1.). When the elution volume was decreased using the same QIAamp Fast DNA Stool mini kit protocol, the DNA concentration increased significantly, but the purity decreased.

Table 4.2.1.: DNA Concentration and Purity using the QIAamp Fast DNA Stool Mini kit

Study no.	QIAamp Fast DNA Stool mini kit			
	200 µl elution volume		60 µl elution volume	
	DNA (ng/µl)	Purity (260/280)	DNA (ng/µl)	Purity (260/280)
3	0.4	0.35	4	5.2
183	0.8	1.49	6.9	3.04
191	0.1	0.11	13.4	2.97
294	0.5	0.65	1.2	1.1

The elution volume was reduced to obtain a higher concentration of DNA.

With the CTAB extraction method (protocol B, Table 4.2.2), except for specimen number 3, the nucleic acid concentration obtained was good, but the purity was not. An electrophoresis gel was done to establish DNA integrity (Fig 4.2.1). Specimen number 191 produced a distinct band, whereas specimen 183 and 294 produced faint bands. Applying the CTAB protocol on formol-ether concentrated stool improved the results for specimen number 3 but not for the others. The PCR with the DNA from specimen number 3 was unsuccessful for both *G. lamblia*e and *C. parvum*.

Table 4.2.2.: DNA Concentration and Purity using the CTAB Method

CTAB Method				
Study no.	Non-concentrated stool		Concentrated stool	
	DNA (ng/μl)	Purity (260/280)	DNA (ng/μl)	Purity (260/280)
3	0	0	20.7	1.78
183	18.1	1.4	0.9	0.95
191	70.9	1.67	4.6	1.6
294	16.1	1.05	11	1.65

The CTAB method was evaluated using both concentrated and non-concentrated stools. The assumption made was that concentrating stool prior to the CTAB extraction will increase the DNA yield. However this table shows otherwise.

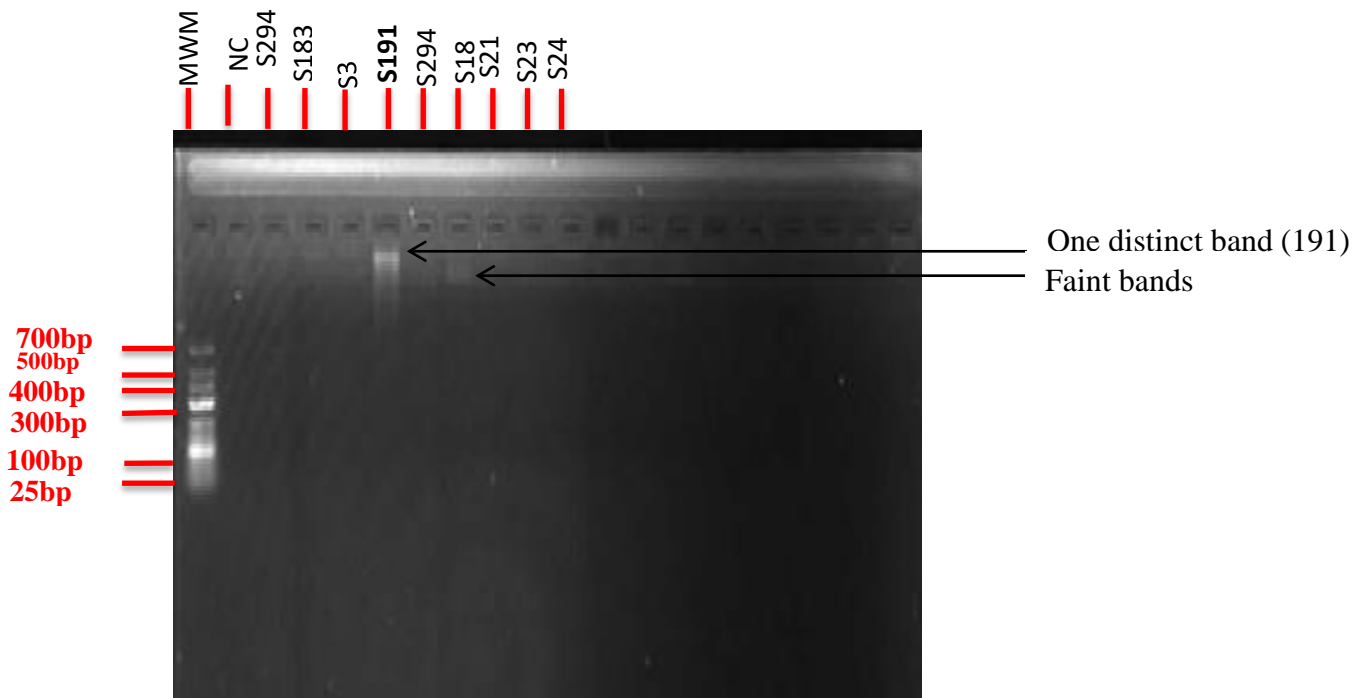


Fig 4.2.1: DNA Extraction a result using the CTAB DNA Method

The molecular weight marker (MWM) is shown on the first lane, on the sixth lane one distinct DNA band can be seen, as well as a faint DNA band in lane 8. NC refers to the negative control

When the QIAamp DNA mini kit protocol for blood specimens (protocol C. Table 4.2.3) was used, good DNA was obtained from only one of the four specimens. Ultimately, use of

the same QIAamp DNA mini kit with the protocol for tissue specimens (protocol D, table 4.2.3) resulted in good quantities of DNA with sufficient purity. However, PCR on these specimens for both parasites produced primer dimers and no product (Fig 4.2.2.).

Table 4.2.3.: DNA Concentration and Purity using the QIAamp DNA Mini Kit

Study no.	QIAamp DNA mini kit			
	Blood protocol		Tissue protocol	
	DNA (ng/μl)	Purity (260/280)	DNA (ng/μl)	Purity (260/280)
3	45.5	2.02	69.6	2.2
183	2.6	2.03	11.6	2.13
191	1.7	4.36	69	2.02
294	0	1.56	10.7	2.31

The protocols for blood and tissue specimens were used because of the different lysis steps, as compared to the stool protocols.

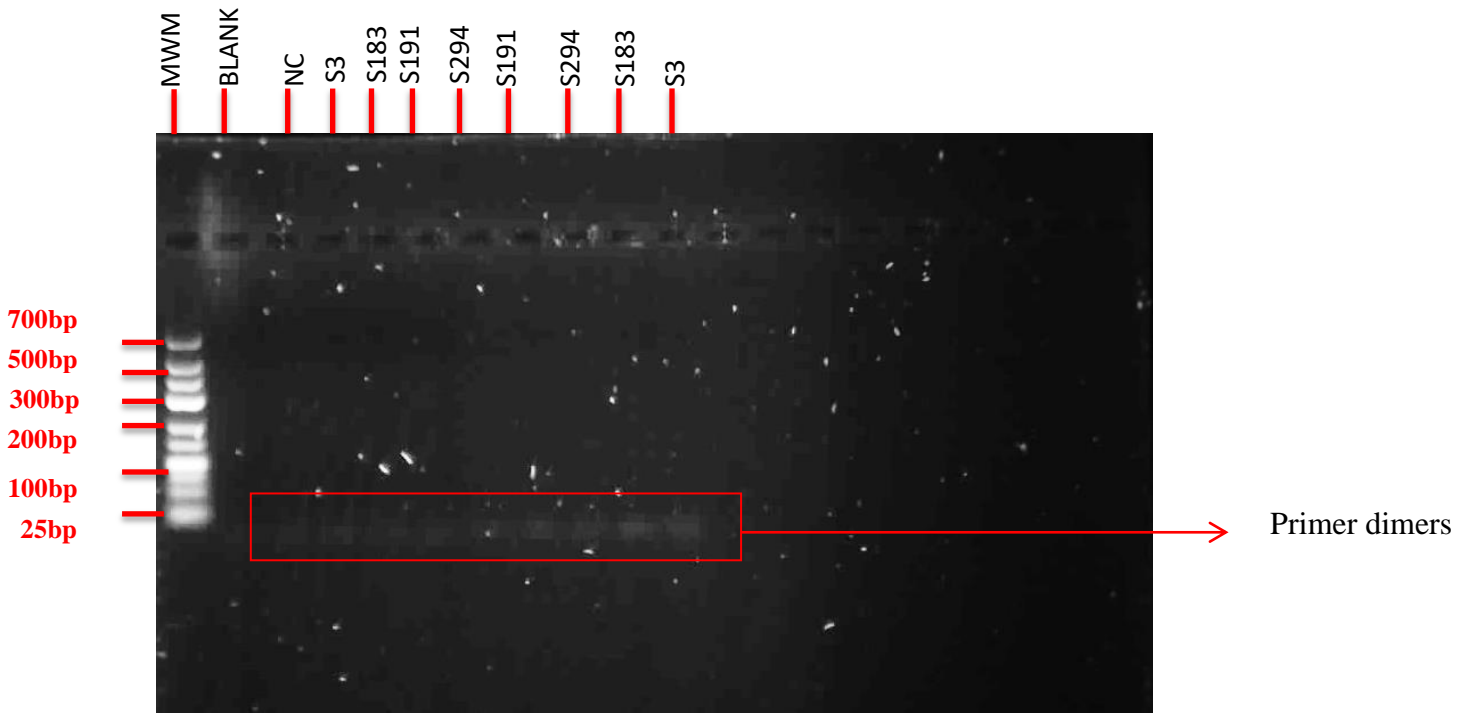


Fig 4.2.2: Unsuccessful PCR using DNA extracted from the tissue protocol of the QIAamp DNA Mini kit

The molecular weight marker (MWM) is shown on the first lane. The specimens used are represented by (S). NC refers to negative control. The red block highlights the primer dimers that formed. The idea behind running this PCR was to get a positive *C. parvum* and *G. lamblia* DNA band, which would thereafter be sequenced. If the sequences were positive the DNA could be used as a control for all the specimens collected.

DNA extraction methodologies were also tested on positive specimens from a private laboratory (Table 4.2.4.). Using a lysis temperature of 100°C for 10 minutes; resulted in DNA that was insufficient in amount and impure. Similar DNA was obtained when an additional step (addition of 180 µl ATL buffer and 20 µl of proteinase K to stool specimens, followed by incubation at 56°C for 3 hours) was implemented before applying the QIAamp Fast DNA stool mini kit protocol (protocol A, Table 4.2.4).

When silica beads were added to the specimens, prior to using protocol A, the DNA obtained remained impure as well as insufficient in quantity. When the QIAamp DNA mini kit protocol for blood specimens (protocol C, table 4.2.4.); was tried, the quantity and quality of DNA obtained was not sufficient for use with PCR. Acceptable quantity and quality of DNA was obtained from specimens PL3 and PL4 (protocol D, table 4.2.4.) however, PCR was not successful. DNA extraction on formol-ether concentrated stool specimens by means of the protocol for tissue specimens (protocol D), rendered poor quality and quantity of DNA. Silica bead pre-treatment on specimens before use of protocol D resulted in a low concentration but good purity for one specimen but no DNA for the other. PCR was unsuccessful on the specimen that had good purity.

Table 4.2.4.: DNA Concentration and Purity using Specimens from a Private

Laboratory

Extraction technique	PL1		PL2		PL3		PL4	
	DNA (ng/μl)	Purity (260/280)	DNA (ng/μl)	Purity (260/280)	DNA (ng/μl)	Purity (260/280)	DNA (ng/μl)	Purity (260/280)
Protocol A: 100°C lysis	0.4	0.55	2.6	3.39	ND	ND	ND	ND
Protocol A with an additional step*	0.4	1.28	1.8	2.26	ND	ND	ND	ND
Protocol A with silica beads	0.3	3.7	0.78	0.2	ND	ND	ND	ND
Protocol C	3.9	3.05	6	2.7	7.8	1.77	10	1.58
Protocol D	5.7	2.18	6.3	2.35	15.9	2.14	17	2.24
Protocol D: concentrated stool	ND	ND	ND	ND	0.6	0.99	0.8	0.72
Protocol D: silica beads	ND	ND	ND	ND	6.2	1.8	0	0

PL1 and PL3: microscopically positive for *C. parvum* in a private laboratory. PL2 and PL4: microscopically positive for *G. lamblia*e in a private laboratory. These specimens were used to obtain control DNA. * An additional step prior to DNA extraction which included adding 180 μl of ATL buffer and 20 μl of proteinase K to the stool specimens and placing this in a 56°C water bath for 3 hours. DNA refers to the DNA concentration that is measured in nanograms per microliter. The purity of DNA was calculated using the 260/280 ratio. ND represents specimens that were not done.

Chapter Five: Discussion

The Ziehl-Neelsen stain in its classic or modified form is usually employed for the detection of acid fast microbes. With this staining technique, it is also possible to detect non-acid fast microbes, which take on the counter stain. Coccidian oocysts stain red against a blue background (Samie *et al.*,2009). The modified trichrome stain is known to detect various protozoa which stain reddish-purple against a dark green or blue background (Shoaib, 2002). The modified Ziehl-Neelsen and the modified trichrome stains were applied in the detection of protozoa listed in Table 2.1.

The key intestinal parasites causing diarrhoea in children aged 0-5 years in the eThekweni district of KZN were identified by microscopy as *Giardia lamblia* and *Cryptosporidium parvum* (Table 4.1.). Both the modified Ziehl-Neelsen and the modified trichrome staining technique, detected a higher number of *C. parvum* as compared to *G. lamblia*. According to the Chi-Square Pearson's test, the higher number of *C. parvum* detected in comparison to *G. lamblia*, was statistically significant ($p < 0.05$). Only two patients were found to be co-infected with both *C. parvum* and *G. lamblia* when both staining techniques were used.

With the modified trichrome stain, *G. lamblia* trophozoites appeared red. The nuclei, axostyle and flagellum of the pear-shaped trophozoite can be clearly seen in Figure 4.1.1. Since cysts of, *G. lamblia* are not acid-fast, those identified by means of the modified Ziehl-Neelsen staining technique, stained blue as part of the background stain (Fig 4.1.3). *C. parvum* oocysts stained red with the modified Ziehl-Neelsen stain (Fig 4.1.2.) and with the modified trichrome stain.

In Ethiopia Adamu *et al* (2006) had found a prevalence of 6.3% for *G. lamblia* and Ansari *et al* (2012), also reported a similar prevalence in Kathmandu, India, of 3.4%, which is concordant with the findings of our study. A study by Wellington *et al* (2008) in Nigeria, reported a prevalence of 27.6% and 4.8% for *C. parvum* and *G. lamblia* respectively, these findings correlate well with the results in this study. Our results for *Cryptosporidium* are also in agreement with Samie *et al* (2009) in the Limpopo province of South Africa, who had found *C. parvum* to be prevalent in 25% of hospital attendees experiencing diarrhoea.

For most of the patients enrolled in this study, no protozoal parasite was detected in stool specimens. This could be attributed to one specimen being collected, instead of multiple stool specimens (Adamu *et al.*, 2006). Parasites are shed intermittently (Adamu *et al.*, 2006); hence at least three stool specimens should be collected on three different days. However, this was not possible in this study as collecting quality stool specimens from these young children was quite challenging. Furthermore, most children were outpatients and not available for repeat specimen collection. Parents were also not keen on the collection of more than one specimen from their child. Non-protozoal causes of the diarrhoea provide another explanation for these negative findings.

Cryptosporidium detection requires the presence of 50 000 – 500 000 oocysts per gram of stool (Tahvildar-Biderouni and Salehi, 2014). To achieve this in as many specimens as possible, concentration of stool specimens is advised prior to staining with the modified Ziehl-Neelsen method. This increases the accuracy for detecting *Cryptosporidium* in stool specimens (Tahvildar-Biderouni and Salehi, 2014). In this study we did not concentrate

stool prior to staining, as the amount of stool specimens collected from most children, was insufficient to perform formol-ether concentration. Hence it is possible that we may have missed a significant proportion of entero-pathogenic protozoa during microscopic examination.

Children under two years were the most affected by *C. parvum* and *G. lamblia* as compared to those children that were between the ages of two-five years (Table 4.1.2.). Our study concurs with the findings of Ansari *et al* (2012), who reported diarrhoea in 69.9% of children less than 2 years. The high prevalence of protozoal infection in children under the age of 2 years in our study could be attributed to formula feed or mixed feed milk. Formula fed infants could be ingesting milk that contains parasites, since contaminated water and unhygienic habits may be used in the preparation of milk (Black *et al.*, 1982). Infants that are breastfed are protected against severe diarrhoea by maternal antibodies. Adequate acquisition of immunity only occurs between 12-16 months of age (Ansari *et al.*, 2012).

There were more females infected with *C. parvum* and *G. lamblia* than there were males (Table 4.1.3.), although this did not reach statistical significance ($p > 0.05$). Of all the children infected with *C. parvum*, a third of them was positive for HIV and had a history of exposure to HIV (Table 4.1.4.). The HIV status of *Cryptosporidium* infected individuals correlates well with other studies that have indicated an association between HIV and *C. parvum*. However, there were more *Cryptosporidium* positive children that were HIV negative as compared to those that were HIV positive. This could be due to the weak immune system of infants which is still developing. In addition, *Cryptosporidium* is an

entero-pathogen capable of infecting both immune competent and immune compromised hosts (Table 2.1.). Therefore children in this study that harboured *C. parvum*, were immune-competent. None of the children infected with *G. lamblia* were HIV positive or had a history of exposure, hence there was no association between HIV and *G. lamblia* infection in this study.

The majority of the children infected with *C. parvum* and/ or *G. lamblia* used water from a communal tap (Table 4.1.5.). However *G. lamblia* cysts and *C. parvum* oocyst are resilient and have the ability to resist chlorination and may still be present in treated water (deRegnier *et al.*, 198 , Johns *et al.*, and 1995 Chauret *et al.*, 1995). Improper sanitation habits facilitate the transmission of entero-pathogenic protozoa. In this study the majority of the caregivers had reported the use of flush toilets in their households (Table 4.1.6.). However, that does not necessarily correlate with good hand hygiene.

More children infected with both *C. parvum* and *G. lamblia* had diarrhoea for one to three days as shown in Table 4.1.7. This suggests most children had acute diarrhoea even though a considerable amount of the infected children were either HIV positive or had a history of exposure to HIV. Some children that were infected with *C. parvum* suffered from malnutrition with signs of kwashiorkor (Table 4.1.8). This was not the case with those infected with *G. lamblia*. However, the numbers are small. Akingbade *et al* (2013) indicated that malnutrition may also be listed as a cause of death in patients with diarrhoea. This is because diarrheic patients eat less; therefore they have a reduced ability to absorb nutrients essential for their survival.

Bright field microscopy is used in many laboratories; however the inability to detect low numbers of oocysts and cysts of *C. parvum* and *G. lamblia* respectively, makes it less sensitive than other diagnostic techniques (Johnston et al., 2003). PCR is considered more sensitive and specific than microscopy (Morgan *et al.*, 1998) with a reported sensitivity of 100% (Elsafi *et al.*, 2013). Hence it was used in this study in an attempt to confirm the microscopic observations.

Extracting DNA from stool specimens posed a challenging task. Various DNA extraction methodologies were tried, tested and manipulated. However none worked. Extraction methodologies were tried on microscopically positive specimens (Tables 4.2.1, 4.2.2 and 4.2.3) but none of the extraction products shown in table 4.2.1 were acceptable for use with PCR. In table 4.2.3 the protocol for tissue specimens produced DNA extracts of sufficient quantity and quality; however PCR was unsuccessful as the presence of primer dimers and the absence of a product were observed (Fig 4.2.2.).

We considered three possible reasons for the lack of success. The specimens we used may not have been true positives, the storage at – 20°C could have negatively impacted or the cyst and oocyst walls could be too strong to lyse with the methods employed. To address the first two possibilities we used specimens kindly provided by one of the private laboratories in Pretoria. These specimens were fresh and were microscopically confirmed positive for *C. parvum* and *G. lamblia*, respectively by experienced technologists. However, only the protocol for tissue specimens produced acceptable DNA (Table 4.2.4). PCR performed remained unsuccessful for both protozoal species, suggesting that the harshness of the cyst walls was the problem.

The QIAamp DNA Mini kit was tried (Table 4.2.3) since the protocol contained lysis steps that in essence were similar to the stool kit protocol, with just one or two variations. Both the protocols for extraction from blood specimens and tissue specimens were tried. Silica beads (Table 4.2.4) were incorporated into the extraction protocols to mechanically disrupt the oocysts and cysts of *C. parvum* and *G. lamblia*, respectively. One extract produced low concentration but sufficient purity whilst the other specimens produced insufficient quality and quantity of DNA.

The CTAB DNA extraction method was tried since it is used to get DNA out of *Mycobacterium tuberculosis*, which is also a difficult organism to extract DNA from. Hence it was assumed that it may work on the robust *C. parvum* oocysts. The formol-ether concentration was performed prior to DNA extraction with the CTAB and tissue specimen protocols (protocols C and D, tables 4.2.2 and 4.2.4, respectively). Sufficient stool was present in specimens that underwent formol-ether concentration. This was not successful (Table 4.2.2 and Table 4.2.4).

Hawash (2014) modified the manufacturer's protocol for use with the QIAamp Stool Mini kit (Qiagen, Hilden, Germany) since their application of the manufacturer's protocol produced a faint PCR band for *C. parvum*. The modification made was lysis at 100°C for 10 minutes. This modification resulted in a clear bright band when PCR was performed. However when the same modification was implemented with the Qiagen protocol in our study (Table 4.2.4.), the DNA extract was of poor concentration and purity. A study conducted by Moore et al (2016) had also reported modifications to the QIAamp Stool Mini kit. These modifications included 5 cycles of freezing and thawing as well as heating

and cooling to weaken the oocyst wall of *Cryptosporidium*. This was done in order to increase the yield of DNA extracts. This suggests that the QIAamp Stool Mini kit is inefficient for the extraction of DNA from entero-pathogenic protozoa, since the yield of DNA is low. The modifications by Moore *et al* (2016) had only been published after laboratory work for this study was completed, therefore we could not have tried these modifications to the QIAamp Fast DNA Mini kit.

The complexity of the oocyst wall described in chapter 2.4.3.2 could have been the primary reason for unsuccessful PCR. The DNA obtained may have been from other microorganisms. Various treatment procedures targeting digestion of the oocyst wall of *C. parvum* were investigated by Harris and Petry (1999). Their findings suggest that proteinase K is most effective in the digestion of the oocyst wall. Trypsin was also effective in digesting the oocyst wall of *C. parvum* while, pepsin was the least effective. In addition, ultra-sonication and phenol-chloroform extraction proved not to be effective in lysing the oocyst wall. All DNA extraction techniques used in the study presented in this dissertation included proteinase K, however it is possible that an insufficient amount, could have prevented digestion of the oocyst wall. The amount of proteinase K used was according to the recommendations supplied by the manufacturer of the kits.

Low PCR efficacy on specimens that had good purity and sufficient quantity could have been affected by the presence of inhibitors such as bilirubins, bile salts, heme and carbohydrates in the faecal specimen (Oikarinen *et al.*, 2009). Inhibitors interfere with the reaction between DNA and DNA polymerase (Tsai and Rochelle, 2001) if co-extracted with the DNA (Schrader *et al.*, 2012). Amplification of nucleic acids can also be inhibited

by unsuccessful lysis of the oocysts and degradation of nucleic acids (Carey et al., 2004), which could possibly also have been the case in this study when finally DNA of sufficient quantity and quality was used.

A recent study in Cambodia, investigating the various species and subtypes of *Cryptosporidium*, demonstrated evidence of both anthroponotic and zoonotic transmission. In addition the authors found multiple species of *Cryptosporidium* involved in causing diarrhoea among children below 16 years of age. The different species of *Cryptosporidium* causing diarrhoea included; *C. hominis*, *C. melagridis*, *C. parvum*, *C. canis*, *C. suis* and *C. ubiquitum*. *C. hominis* was the highest cause of diarrhoea, followed by *C. melagridis* and then *C. parvum*. *C. canis*, *C. suis* and *C. ubiquitum* were present in one stool specimen, respectively. Furthermore, the authors also indicated that 50% of *C. parvum* infected children had *C. parvum* with subtype IleA7G1 present. This subtype is rare, with only a single strain being previously described in India (Moore et al., 2016). The possibility of other species and subtypes of *Cryptosporidium* present in KwaZulu-Natal, South Africa, cannot be excluded. Since these findings demonstrate that various species and subtypes exist. This could be a possible reason for the unsuccessful PCR described in this dissertation since the primers targeted specifically *C. parvum*.

Chapter Six: Conclusion

This study investigated the role of entero-pathogenic protozoa in causing diarrhoea in children between the ages of 0-5 years. The species found were *Cryptosporidium parvum* and *Giardia lamblia*. Of these parasites, *Cryptosporidium parvum* demonstrated a higher prevalence than *Giardia lamblia*. *Giardia lamblia* infection has been strongly associated with children under the age of five that attend day care centres in KZN. However our study demonstrates a stronger association between *C. parvum* infection and children under the age of five. *C. parvum* is known to infect both the immune compromised and immune competent host. This study reports that diseases other than HIV infection could have impacted on the immunity of parasite-infected children. Children experiencing diarrhoea should also have their stool examined for parasites, in addition to bacteria and viruses, which are not always done, hence intestinal parasites are missed, and continue to proliferate among children in the eThekweni district of KZN.

Apart from microscopy, PCR diagnosis was tried but this was unsuccessful. Microscopy is known to be less sensitive than PCR, but highly specific if verified by an independent, experienced laboratory technician. Most laboratories in developing countries continue to use microscopy in the diagnosis of intestinal parasitic infections. Even though a substantial amount of evidence highlights the insensitivity of this technique. This is because reliable techniques such as PCR are expensive and require special equipment as well as skilled laboratory workers. Furthermore, conducting PCR on stool specimens aimed at targeting intestinal parasites remains a challenge as indicated in this dissertation.

With the collection of multiple stool specimens, incorporation of suitable concentration techniques, observations by an experienced microscopist and quality control conducted by an independent laboratory technologist, microscopy can still be used as a diagnostic tool for the identification of intestinal parasites. This study was limited to a study population of children from one hospital in KZN and is therefore not representative of the entire population of children in KZN. Future studies should aim to investigate multiple cohorts of children across KZN.

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Appendix 1: BREC Approval Letter



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03 March 2015

Prof P Moodley
719 Umbilo Road Congella
moodleyp@ukzn.ac.za

Dear Prof Moodley
PROTOCOL: Provincial Surveillance for Hospital Acquired Infections and
Outbreak Investigation. REF: BE222/13.

We wish to advise you that Ms Nakita Mathaparsadh's study for degree purposes (MSc) "*intestinal parasites causing diarrhoea in children aged less than five years in the Ethekwini district of KZN*" has been noted and approved by the sub-committee of the Biomedical Research Ethics Committee under the above (BE222/13) ethics approval.

This approval will be ratified by a full Committee at its next meeting taking place on 14 April 2015.

Yours sincerely

Mrs A Marimuthu
Senior Administrator Biomedical Research Ethics Committee

cc: N Mathaparsadh nakita236@gmail.com
Dr ZL Kwitshana Kwitshana@ukzn.ac.za

Appendix 2: BREC Approval of Amendments



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17 August 2015

Prof P Moodley
719 Umbilo Road Congella
moodleyp@ukzn.ac.za

Dear Prof Moodley
PROTOCOL: Provincial Surveillance for Hospital Acquired Infections and
Outbreak Investigation. REF: BE222/13.

We wish to advise you that the amendment request from Ms Nakita Mathaparsadh in relation to her study for degree purposes (MSc) has been noted and provisionally approved by the sub-committee of the Biomedical Research Ethics Committee subject to postgraduate approval.

This approval will be ratified by a full Committee at its next meeting taking place on 14 April 2015.

Yours sincerely

Mrs A Marimuthu
Senior Administrator Biomedical Research Ethics Committee

Appendix 3: Study Information

Information to Patients

Title: Surveillance for Diarrhoeal Pathogens in Children 5 years and below in KZN

We are a team of doctors, nurses and scientists. My name is..... and I work in the Department of Infection Prevention and Control, University of KwaZulu-Natal / King Edwards Hospital. We are involved in conducting healthcare associated research for the KwaZulu-Natal Department of Health so that we may better understand diarrhoeal disease. Diarrhoea is one of the most common causes of illness and death in children aged 5 years and below. Diarrhoea can be caused by germs that are passed to a child through food or water or from other people.

At the moment we are studying what causes diarrhoea and which medicines will best treat it. This is in order to improve treatment as well as learn better ways to prevent this illness. I invite you to consider allowing your child/ward to participate in this study that involves children 5 years and below with diarrhoea. Your child/ward having diarrhoea means that he/she has passed three or more loose, liquid or watery stools or at least one bloody loose stool than normal within a day. In order to treat your child/ward for this illness, sometimes a stool specimen and blood specimen is taken.

There is also a question on whether you know the HIV status of your child/ward. You may choose not to answer this question if you feel uncomfortable doing this. If you do not know your child's HIV status and you want to know, we can request that, an HIV test is done by

the doctor treating your child. This is to better manage the disease. If you do not want to know the HIV status of your child/ward, then we do not have to test him/her. However you may also agree that we can take your child's/ward's blood for HIV testing, but we will not disclose his/her result to anyone, but use it for the study only.

The care that your child receives will not, be affected by you agreeing or refusing to take part in the study. The people working on this study are separate from the staff at this clinic/hospital.

All the information that we get from you on behalf of your child/ward will be kept confidential at all times. If you agree to take part in the study your child will be given a study number. Only this study number will be used on study records and blood/stool samples, therefore people working in the laboratory or people looking at your child/ward results (apart from his/her doctor) will not be able to link him/her or his/her name to the results.

Specimen collection:

The nurse will assist in collecting the stool specimen into a leak-proof clean dry container. A teaspoon of blood specimen will also be collected into a tube from your child/ward. The stool specimen will be sent to test for the presence of common germs. We will test his/her blood for Antibody levels against Rotavirus and other germs. Taking these specimens will not harm your child/ward in any way, but he/she may experience minor discomfort.

You will be required to spend about 30 minutes of your time in the clinic/hospital so that we will be able to interview you and collect these specimens from your child/ward. Taking part in the study will not put your child/ward at any risk. The participation of your child/ward will benefit us in understanding the causes of diarrhoea and knowing the best treatment for children like your child/ward. If the findings from our laboratory investigations require an additional treatment for your child/ward condition we will inform the doctor treating your child. If your doctor feels that it is necessary he/she will contact you personally so that your child/ward can be treated with a new medication. This might cost you one extra visit to the clinic/hospital.

You will not receive any money for agreeing to allow your child/ward to participate in this study.

If you agree to allow your child/ward participate in this study, we require that you sign a form which gives us permission to:

- Interview you and record data on an information sheet
- Collect specimens from your child/ward for laboratory tests
- Get your child's/ward's HIV status from you (not compulsory)

This study has been ethically reviewed and approved by the UKZN Biomedical research Ethics Committee (approval number.....). In the event of any problems or

concerns/questions you may contact Prof P Moodley or the UKZN Biomedical Research Ethics Committee, contact details as follows:

Prof P Moodley

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Do you have any questions or do you need clarity on any aspect?

Thank you for your time.

Appendix 4: Questionnaire

Patient no. _____
 Date of birth (dd/mm/yy) _____
 Date of admission _____
 Date of specimen collection _____

No specimen

Is the stool

watery	
bloody	
unknown	

Age _____

Gender _____

Weight _____ kg _____ centile

Height _____ cm _____ centile

Temp _____ C

HIVstatus On ART?
 CD4 count/percentage

P	N	Exposed	Unexposed	Unknown
Y	N			

How long does the patient have diarrhoea for?

Is the child underweight for age °	Y	N
Does the child have kwashiorkor °	Y	N

Has the child received all immunizations to date:

Vaccine Date received

Birth	BCG	
6 wks	OPV	
	RV (1)	
	DTaP-	
	Hep B (1)	
	PCV (J)	
10 wks	DTaP-I	
	Hep B (2)	
14 wks	RV (2)	
	DTaP - I	
	Hep B (3)	
	PCV (2)	

9 mnth	Measles (1)	
	PCV (3)	
18 mnths	DTaP-	
	Measles (2)	

How many doses has of vitamin A has the child received _____

Has the child received Zinc supplementation?

UNKNOWN

YES _____ NO _____

Has the child received anti helminths?

If child <6 months, is the child

exclusively breastfed	
formula-fed	
mixed fed	

What is the water supply at home

Piped water in dwelling	
Piped water on site	
Neighbour's tap	
Communal tap	
Rainwater	
Borehole on site	
Borehole off site	
River/stream/flowing	
Dam/pool/stagnant water	
Well	
Spring	
Other	

Specify _____

Type of sanitation

Flush	
VIP	
Pit	
Other	

Specify _____

Availability of soap to wash hands

Always	
Sometimes	
Never	

Current treatment

Any treatment prior to coming to this clinic /hospital

Appendix 5: Informed Consent

Consent Form

The study has been explained to me in detail and I grant permission for stool specimen to be collected from my child/ward. The stool specimen will be examined for germs causing diarrhoea and medicines that may kill these germs.

Yes..... No.....

I give permission for the doctor/nurse to record the HIV status of my child/ward on the information sheet and I understand that this information will not be linked to my his/her name.

Yes..... No.....

I grant permission for a blood specimen to be collected from my child/ward and examined for HIV and antibody levels against some germs that cause diarrhoea.

Yes..... No.....

Name of Parent/Guardian:

Name of Doctor/Nurse

Signature-----

Signature-----

Date: _____

Name of Translator (if applicable)

Signature-----

NB: This sheet is to be removed by the study co-ordinator upon completion of enrolment and delivered in a sealed envelope to the principal investigator. The forms will be stored in a separate, locked cabinet and destroyed when the study is officially closed