Detection of *Cryptosporidium* Species in Kenya Using Lateral Flow Loop-Mediated Isothermal Amplification

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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DEDICATION

I dedicate this thesis to my loving parents Joseph Mamba and Rosemary Olesi for their continued guidance and support.

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First and foremost, I would like to thank the Almighty God for seeing me through this study to its final conclusion.

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LIST OF ABBREVIATIONS AND ACRONYMS

BIP: Backward Inner Primer

DNA: Deoxyribonucleic Acid

FIP: Forward Inner Primer

HAART: Highly Active Antiretroviral Therapy

HIV/AIDS: Human immunodeficiency Virus/ Acquired Immunodeficiency Syndrome

LAMP: Loop-mediated Isothermal Amplification

LFD: Lateral Flow Dipstick

LP: Loop Primer

N-PCR: Nested Polymerase Chain Reaction

PCR: Polymerase Chain reaction

RT-PCR: Reverse Transcriptase Polymerase Chain Reaction

SAM -1: S-Adenosylmethionine synthase -1

SBP: Stem Backward Primer

SP: Stem Primer

SS rRNA: Small Subunit Ribosomal Ribonucleic Acid

USFDA: United States Food and Drug Administration

ABSTRACT

Cryptosporidium is a protozoan parasite and a major cause of diarrhea in children and immunocompromised patients. Current diagnostic methods for cryptosporidiosis such as microscopy depict inter-observer variability and do not allow for batch processing while techniques such as PCR indicate higher sensitivity levels, but are seldom used in developing countries due to the associated cost. This study aimed to develop a more sensitive lateral flow dipstick (LFD) LAMP test based on SAM-1 gene and with the addition of a second set of reaction accelerating primers (stem primers). A loop-mediated isothermal amplification (LAMP) technique, a method with shorter time to result and with equal or higher sensitivity compared to PCR, has been developed and applied in the detection of *Cryptosporidium* species. The detection limit was determined using analytical sensitivity based on reference DNA, sensitivity was achieved using archived samples, while specificity was achieved using closely related DNA. This test has a detection limit of 10pg/ µl (~100 oocysts/ml) suggesting need for more sensitive diagnostic tools. The stem LFD LAMP test showed analytical sensitivity of 10 oocysts/ml compared to 100 oocysts/ml (10 pg/µl) for each of the SAM-1 LAMP test and nested PCR. The stem LFD LAMP, SAM-1 LAMP and nested PCR detected 29/39 (74.3%), 27/39 (69.2%), and 25/39 (64.1%) positive samples of previously identified *C. parvum* and *C. hominis* DNA, respectively. Using the 67 *Cryptosporidium* DNA clinical samples, the stem LFD LAMP detected 16/67 (23.8%) positive samples, SAM-2 LAMP detected 14/67 (20.8%) positive samples, while the nested PCR detected 11/67 (16.4%) positive samples. The 67 samples had not been sequenced and had not been tested to determine whether they were positive or negative. The positive samples may be a representation of the prevalence of the disease in the population. Pre-heating the templates increased detection by stem LFD LAMP to 19 samples. Time to results from master mix preparation step took ~80 minutes. The test was specific (100%), and no cross-amplification was recorded with non-target DNA. This stem LFD LAMP test is more appropriate for detection of *C. hominis, C. parvum* and *C. meleagridis* DNA in human stool samples. It can be used in algorithm with other diagnostic tests and may offer promise as an effective diagnostic tool in the control of cryptosporidiosis.

Key words: LFD Stem LAMP SAM-1, PCR, SAM-1 LAMP, N-PCR

CHAPTER ONE

1.0 INTRODUCTION

Cryptosporidiosis a disease caused by a large number of phenotypically and genotypically diverse *Cryptosporidium* species that present major public health concern (Karanis *et al.*, 2007). The enteric pathogen *Cryptosporidium* was first identified as a human pathogen in 1976 when a three year old child was reported to have diarrhea characterized by a self-limiting enterocolitis (Meisel *et al* ., 1976; Iqbal *et al* ., 2012). However, with the emergence of the HIV pandemic in the 1980s, *Cryptosporidium* became a widely recognized human pathogen. The first case of a HIV co-infection with cryptosporidiosis was reported in 1982 with many more reports on cryptosporidiosis being filed, thus, becoming a significant pathogen in HIV/AIDS infections. Several routes of transmission of cryptosporidiosis in humans are known and they include person to person, food borne, waterborne, and Zoonotic (Coupe *et al.*, 2005).

Cryptosporidium hominis and *Cryptosporidium parvum* are the most common species that cause disease in humans (Coupe *et al* ., 2005; Hadfield *et al* ., 2011; Mary *et al* ., 2013; Widerstrom *et al* ., 2014). However, other species such as *C. meleagridis, C. canis, C. muris, C. ubiquitum, C. suis, C. andersoni, C. cervine* and *C. felis* may occasionally cause diarrhea in humans (Coupe *et al* ., 2005; Nichols, *et al* ., 2010; Rafiei, *et al* ., 2014). *Cryptosporidium* causes disorders of the respiratory and digestive systems resulting in poor health and economic losses. Cryptosporidiosis is a chronic and often life-threatening disease in immunocompromised patients as it causes an acute self limiting infection of the gastrointestinal tract (Moghaddam *et al* ., 2008; Mary *et al* ., 2013). This protozoan pathogen has been implicated to cause disease under three epidemiological conditions which include sporadic [often transmitted by water] outbreaks of self-limiting diarrhea affecting healthy people; diarrhea and malnutrition affecting young children; and chronic life threatening disease among immunocompromised persons mostly HIV/AIDS cases (Siobhan & Tzipori, 2008). Morbidity and mortality resulting from cryptosporidiosis infection has been reduced to a

bare minimum in industrialized nations through adherence to highly active antiretroviral therapy (HAART) (Wang *et al* ., 2012; Adamu *et al* ., 2014). However, in developing countries lacking effective HAART programs, cryptosporidiosis continues to pose a major threat to immunocompromised persons (Adamu *et al* ., 2014) including preschool and school going children whose immunity is not fully developed (Mbae *et al* ., 2013).

1.2 Epidemiology of cryptosporidiosis

Effective HAART programs have served to reduce the incidences of cryptosporidiosis in developed countries, but recent reports show outbreaks resulting in a slight increase in prevalence in Sweden, Australia, US and UK (Widerstrom *et al* ., 2014; Lal *et al* ., 2015). In 1993 a major outbreak of Cryptosporidiosis in Wisconsin, US occurred where more than 403,000 people were affected, with over 5,000 confirmed cases, and hundreds of deaths resulted from this infection (Iqbal *et al* ., 2012). In the US, increased incidence of cryptosporidiosis has been attributed to several factors including better reporting, better diagnostic tests, increased awareness or an actual increase in the incidence (Yoder & Beach, 2010). A review of global outbreaks involving waterborne protozoan parasites between 2004 and 2010 revealed that there were at least 190 outbreaks with 46.7% occurring in Australia, 16.5% in Europe and 30.6% in North America. More specifically, *Cryptosporidium* outbreaks account for 60.3% of the total outbreak cases with *Giardia lamblia* accounting for 35.2% and other protozoan infections accounting for 4.5% (Baldursson & Karanis, 2011). In developing countries, cryptosporidiosis is one of the major causes of childhood diarrhea (Gatei, 2006; Molloy *et al*., 2010) where the prevalence ranges from about 10% to 45% in immunocompromised persons (Siobhan & Tzipori, 2008; Muchiri *et al.*, 2009; Mbae *et al.*, 2013). In cases presenting with diarrhea, the prevalence was 8% to 19%, with a significant effect on mortality (Gatei *et al.*, 2006; Tellevik *et al* ., 2015). Further, cryptosporidiosis has been associated with impaired physical fitness, growth and cognitive disorders (Desai *et al* ., 2012). Reported cases of *Cryptosporidium* infections have been on the rise in South Africa (Omoruyi *et al* ., 2014). According to a study that was conducted in Uganda to determine the

prevalence of *Cryptosporidium* infections in children presenting with persistent diarrhea, 73.6% of children infected with HIV had cryptosporidiosis, while 5.9% of HIV negative children were infected with cryptosporidiosis (Tumwine *et al* ., 2005). In Kenya, findings from a recent study in Bungoma County indicated the prevalence of cryptosporidiosis to be 4% with higher infection prevalence at 5.2% in children aged 13-24 months (Kutima *et al*., 2015). Approximately 6.2% of the Kenyan adult population is infected with the HIV virus with cryptosporidiosis being cited as the most prevalent enteric pathogen (34%) within this group (Wanyiri *et al* ., 2014). Further, among the HIV infected persons, findings from a Kenyan study involving HIV infected patients revealed that cryptosporidiosis is common in patients presenting with and without diarrhea (Wanyiri *et al* ., 2014). Among this group, 40% of those who die experience diarrhea with *Cryptosporidium* being a leading indicator of death among HIV/AIDS patients. *Cryptospridium parvum* has been reported as the dominant species in developed countries affecting both humans and livestock (Molloy *et al* ., 2010). However, in developing countries, *C. hominis* has been cited as the dominant species affecting both adults and children (Snelling *et al* ., 2007; Molloy *et al* ., 2010; Squire and Ryan, 2017). Epidemiological studies in Kenya also indicate that *C. hominis* is the dominant species affecting children followed by *C. parvum and C. meleagridis* (Gatei *et al* ., 2006; Mbae *et al*., 2015).

1.3 Diagnosis of cryptosporidiosis

Laboratory-based diagnosis of Cryptosporidiosis within hospital settings can be difficult because majority of diagnosis is based on microscopy, which may not easily detect infections with a small number of oocysts in the sample (CDC, 2015). The undiagnosed nature of cryptosporidiosis coupled to high prevalence of HIV and AIDS especially in developing countries (Morgan *et al.*, 2000), creates the need for development of sensitive and specific diagnostic tools for detection of *Cryptosporidium* species. Initially, microscopic examination of endoscopic biopsies and necropsies was used in the diagnosis of *Cryptosporidium* (Tzipori *et al.*, 1980*;* Weber *et al*., 1991). Following

the discovery of *Cryptosporidium* oocysts in human stool in 1980s, a number of stool concentration techniques, methods for staining, and antigen detection assays were developed using stool samples (Weber *et al.*, 1991). Some of the most commonly used diagnostic techniques include acid-fast staining and fluorescein-tagged monoclonal antibody technique. However, these techniques are time consuming, depict wide variations in sensitivity, and are unable to distinguish between *Cryptosporidium* species (LeChevallier *et al* ., 2003). Additionally, microscopic examination of stool samples is tedious and does not allow batch processing, and requires skilled personnel to identify the approximately 4 - 6µm oocysts (Newman *et al* ., 1993; Omoruyi *et al* ., 2014). Several antibody-based tests have been developed to aid in the diagnosis of cryptosporidiosis. Among them are antigen ELISA - RIDASCREEN® (R-Biopharm, Darmstadt, Germany) for *C. hominis* and *C. parvum* in human stool and RIDAQUICK® (R-Biopharm, Darmstadt, Germany) an immune-chromatography test for *C. parvum*. These tests are easy to perform but indicate poor sensitivity (88%, 82% respectively) especially when compared to molecular-based diagnostic techniques such as PCR (98.9%) (Morgan et al., 1998; Weitzel et al., 2006). Molecular-based diagnostic techniques have been developed that have made it possible to detect, analyze, and identify the different species of *Cryptosporidium*. Morgan *et al.* (2000) developed and described the first PCR based tool for the detection and differentiation of *C. hominis* and *C. parvum*. Since then, other genotyping diagnostic tools such as real-time PCR, restriction fragment length polymorphism, melt curve analysis, and single strand conformation polymorphism analysis have been developed. Among these, real-time PCR recorded the highest sensitivity and specificity values at 100% and 99.1% respectively (Hadfield *et al* ., 2011). Thus, PCR based diagnostic tools have been widely adopted in laboratories for the detection of *Cryptosporidium* species (Cheun *et al* ., 2013; Mary *et al* ., 2013).

Despite good progress achieved with PCR, the technique is still limited to laboratory use due to cost implications relating to the cost of thermocycler and personnel. Therefore,

technologies that could afford the sensitivity and specificity depicted by PCR and are applicable in the field are urgently needed. For instance, Loop-mediated Isothermal Amplification (LAMP) of DNA amplifies DNA using enzymes with strand displacement activities (Notomi *et al.,* 2000). Unlike other molecular tests, LAMP is simple to perform and reported sensitivities that are similar to PCR (98%) using the same gene target (Karanis *et al.,* 2007). Moreover, this method is robust i.e. it can amplify DNA from partially processed or non-processed samples (boiled or native samples), therefore DNA extraction using commercial kits is not necessary (Njiru *et al.,* 2008). The LAMP test gives results within an hour, supposedly has higher specificity since 4-6 primers are used recognizing four to six regions of the target DNA sequence (Mori *et al* ., 2001) and amplification can be achieved using an affordable heating device, such as water bath or heating block. Further, LAMP method forms varied by-products such as magnesium pyrophosphate (white precipitate) (Nagamine *et al* ., 2002) and double-stranded (ds) DNA (Notomi *et al* ., 2000) allowing different detection formats (Parida *et al* ., 2008). Therefore, LAMP offers promising diagnostic technique potential for the detection of *Cryptosporidium* oocysts.

1.3.1 Recent developments in LAMP diagnosis

LAMP tests for *Cryptosporidium* oocysts have been developed and evaluated. LAMP has a high sensitivity (100% compared to 43.75%) and could be used in the detection of *Cryptosporidium* oocysts in epidemiological studies (Koloren et al., 2011; Karanis *et al* ., 2007). Previous findings reported difficulty in duplicating LAMP analytical sensitivity and specificity levels in the field due to technical skills required (Njiru, 2008). Additionally, current formats that use six to eight primers increase chances of forming primer dimers, hence resulting in of false positives. The situation is further complicated by LAMP detection formats such as SYBR green that are non-specific making it difficult to confirm the suspected false results (Njiru, 2012). Other reports showed that LAMP test specificity can be improved through the use of a sequence probe in a dipstick format (Puthawibool *et al.,* 2009).

In this study, a more sensitive and specific LAMP test for *Cryptosporidium* oocysts was designed by employing additional set of reaction accelerating primers in addition to loop primers (Gandleman *et al*., 2011) and by omitting outer primers. This has been successfully done for African Human Sleeping Sickness and Buruli ulcer disease (Njiru – personal communication) and for HIV LAMP test (Gandleman *et al.,* 2011). This should hypothetically increase LAMP sensitivity by up to 100- fold (Gandleman *et al* ., 2011). Moreover, to improve the test specificity, the reactions were carried out with fewer primers and a specific DNA sequence probe in a dipstick format was also used (Njiru, 2011).

1.4 Statement of the problem

Cryptosporidiosis is a chronic and often life-threatening disease in immunocompromised patients, such as HIV/AIDS patients where it causes an acute diarrhea (Coupe *et al.,* 2005). It is also prevalent in animals. The burden of HIV & AIDS is the greatest in Sub Saharan Africa (Ortbald *et al* ., 2013). In Kenya, for instance, the burden of HIV & AIDS stands at more than 60,000 deaths and 100,000 new infections annually (Birx, 2013). In this context, cryptosporidiosis poses a significant threat to the public health system. This problem is exacerbated by the lack of highly sensitive and specific diagnostic tests that can be used in diagnostic and disease surveillance programs and the lack of a reliable and defined treatment approach for the management of cryptosporidiosis. Currently, diagnosis of cryptosporidiosis in Sub-Saharan Africa, especially in the field, relies on microscopy. Microscopy has been found to have lower sensitivity (83.7%) compared to molecular-based tools such as PCR (98.9%) creating a need for better diagnostic tests that have higher sensitivity (Morgan *et al.,* 1998; Chalmers *et al* ., 2011). In the absence of a highly sensitive, specific, reliable, cost-effective, and field applicable test, quantifying the disease burden would be difficult. This would, in turn, pose a challenge to the development and implementation of effective disease control programs by the government.

1.5 Justification

Cryptosporidiosis is a disease of major public health concern. In Kenya, a study conducted in Nairobi involving school going children revealed that approximately 25% of children presenting with diarrhea were infected with at least one intestinal parasite. Of all the positive intestinal parasite cases, 30.5% of the cases were *Cryptosporidium*, with *Entamoeba histolytica* (36.7%), *Giardia lamblia* (16%) being the other major prevalent intestinal parasites (Mbae *et al*., 2013). To aid with diagnosis, standard tests such as formal-ether concentration, and modified Ziehl Neelsen staining techniques have been employed. These diagnostic methods pose a challenge due to high test-to-test variability and inability to facilitate batch processing. Other more sensitive and specific diagnostic methods have been developed like PCR (98.9&) (Morgan et al., 1998). However, they are expensive and their use has been largely limited to the laboratory. With the increasing cases of cryptosporidiosis, a field applicable diagnostic tool with high sensitivity and specificity is desirable. A diagnostic tool based on LAMP technology offers a potential solution to the diagnosis of cryptosporidiosis. LAMP is an easy to perform test, quick, highly adaptable to field conditions, uses a variety of formats to display by-products (which increases visualization), and is a robust test. This study developed a sensitive Lateral Flow Dipstick (LFD) stem LAMP test based on the SAM-1 (S-adenosylmethionine synthase 1) gene that will contribute towards improved diagnosis of Cryptosporidiosis in Kenya. Improved diagnosis will enable the Ministry of Health to apply effective disease control measures that will reduce the prevalence of the disease. Such a reduction will be accompanied by better quality of life, especially among vulnerable patients and reduced disease burden to the economy.

1.6 Objectives

1.6.1 General objective

To develop a modified lateral flow dipstick format loop-mediated isothermal amplification (LAMP) test for detection of *C. parvum, C. meleagridis, and C. hominis* in stool samples.

1.6.2 Specific objectives:

i. To develop a lateral flow dipstick (LFD) stem LAMP test based on SAM-1 (S-adenosylmethionine synthase 1) gene of *Cryptosporidium* spp. and compare the analytical sensitivity of the LFD stem SAM-1 LAMP test to that of the SAM-1 LAMP test and nested PCR using reference DNA (Microbiologics, Minnesota, USA).

ii. To evaluate the sensitivity and specificity of LFD stem SAM-1 LAMP test using clinical samples and compare with SAM-1 LAMP test and nested PCR. Closely related DNA was used to determine the test's specificity.

    Closely related DNA is DNA from other protozoans whose sequence is almost similar to that of *Cryptosporidium* spp. while reference DNA is a standard Cryptosporidium positive DNA sample with known concentration.

1.6.3 Research questions

i. Will the LFD Stem SAM-1 LAMP test achieve higher analytical sensitivity and specificity than the SAM-1 LAMP test and nested PCR?

ii. Will the LFD Stem SAM-1 LAMP test achieve higher sensitivity and specificity than the SAM-1 LAMP test and nested PCR using clinical samples?

1.6.4 Hypothesis

i. The LFD Stem SAM-1 LAMP test will achieve higher analytical sensitivity and specificity than the SAM-1 LAMP test and nested PCR

ii. The LFD Stem SAM-1 LAMP test will achieve higher sensitivity and specificity than the SAM-1 LAMP test and nested PCR using clinical samples.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Introduction

The genus *Cryptosporidium* is composed of protozoan parasites that infect the GIT of all vertebrate classes. More specifically, the parasite infects the microvilli of the epithelial cells of the intestinal villi (Xiao *et al.,* 2004). Different species of *Cryptosporidium* infect the stomach while other species infect the intestines. They have a worldwide distribution where they infect different species of animals. Some species of *Cryptosporidium* infect only one host, while others infect a wide range of hosts (Fayer & Xiao, 2007). Currently, there are 18 known species of *Cryptosporidium* worldwide (Chalmers, 2014) (Table 2.1). Additionally, the different species of *Cryptosporidium* display varying levels of pathogenicity with the severity and duration of infection being dependent on the immune status of the individual. Immunocompetent persons may suffer a mild, moderate or severe acute self-limiting illness, while the immunocompromised persons suffer a severe chronic illness that may result in death in some cases (Fayer & Xiao, 2007).

2.2 General biology of *Cryptosporidium*

2.2.1 Taxonomy

Cryptosporidium belongs to the phylum Apicomplexa, class Sporozoasida, subclass Coccidiasina, order Eucoccidiorida, family Cryptosporidiidae, genus *Cryptosporidium* (Vohra et a;., 2012). Members of the genus *Cryptosporidium* parasitize over 150 species of mammals humans included (Chalmers, 2014).

Prior to the development of current molecular-based research and diagnostic tools, classification of organisms into various genus and species was based on the morphological attributes of the organism as visualized by microscopy (Franco-Duarte, 2019).

Table 2.1: *Cryptosporidium species* and the associated hosts (Chalmers 2014)

Cryptosporidium Spp.	**Major Host**	**Infection in Humans**	**Transmission routes to human**
C. bovis	cattle	Yes - very rare	Contact with cattle
C. canis	dog	Yes- occasional	Contact with dogs
C. cuniculus	Rabbit	Yes- occasionally	Environmental contact,
C. fayeri	Marsupials	Yes – very rare	Contact with marsupials
C. felis	cats	Yes - occasionally	Contact with cats
C. hominis	humans	Yes - frequently	Anthroponotic pathways – direct (human to human) or indirect through water food, fomites etc
C. macropodum	Eastern Gray kangaroo	No	
C. parvum	Humans & ruminants	Yes - frequently	Zoonosis could be direct through contact with animals, or indirect through contaminated water, food, recreational water etc person to person transmission also occurs.
C. ryanae	Cattle	No	
C. scarofarum	Pig	Yes - rarely	Contact with pigs
C. andersoni	Cattle	Yes - rarely	Unknown
C. fragile	Toads	No	
C. moinari	Fish	No	
C. muris	Mice	Yes-rarely	Not known
C. serpentis	Snakes	No	
C. baileyi	Chickens	No	
C. galli	Birds	No	
C. meleagridis	Turkeys	Yes-frequently	Sporadic cases

However, with the advent of new molecular tools such as gene sequencing, PCR, phylogenetic analysis software etc, genotyping and serotyping was greatly enhanced with many organisms being classified correctly (Xiao *et al.*, 1999; Jex *et al.*, 2008). These tools made it possible to classify organisms beyond the species level. In this regard, the small subunit ribosomal RNA gene (18S) has been of great value in the taxonomic classification of *Cryptosporidium*. Other gene targets used include the 70 KD heat shock proteins and the 60KD glycoprotein (Jex *et al.,* 2008). These molecular-based diagnostic tools require a very small number of oocysts, and with rapid processing times, have helped generate gene species-specific unique data that has been used in the classification of *Cryptosporidium* beyond the genus level (Koonin & Galperin, 2003). It is notable that morphological differences that exist between various species of *Cryptosporidium* are not sufficient for species differentiation, and slight differences of the base pairs of the 18S rRNA gene of *Cryptosporidium* species have been invaluable in the taxonomic classification of *Cryptosporidium* species (Fayer & Xiao, 2007).

2.2.2 Morphology of *Cryptosporidium* spp.

The morphology of the parasite varies depending on the stage in the life cycle. The exogenous form, oocysts, are ovoid in shape and have smooth surfaces. The ooocysts are surrounded by a thick wall that has a cleft on one side where the sporozoites are released once suitable conditions for excystationare reached. The size of the developmental stages varies with oocysts estimated at 5×7µm compared to 5×0.5µm for sporozoites, and 1.25µm for trophozoites. Type I and II meronts vary between 1.5 and 3.5 µm. sporozoites have a rough surface, a rounded posterior and a pointed apical region. Trophozoites and meronts have smooth surfaces. Type 1merozoites have a pointed apical region, rough surface and are rod-like in shape. Type II merozoites have a rough surface and a round shape. Microgametes originating from Type II merozoites are 0.1µm in size and also have a rough surface and a spherical shape. The macrogamont is ovular in shape with a rough surface and measures 4×5µm (Microscope Master, 2020).

2.2.3 Life cycle and transmission of *Cryptosporidium* Spp.

Transmission of *Cryptosporidium* is through the fecal-oral route (Gerace *et al* ., 2019). Some species of *Cryptosporidium* such as *Cryptosporidium parvum* are zoonotic and affect a wide range of mammals, including humans (Pumipuntu & Piratae, 2018). Close contact with infected livestock, wildlife, and other infected humans increases chances of infection (Fayer & Xiao, 2007). Contaminated drinking water or recreational waters are transmission vehicles between different hosts. The life cycle of *Cryptosporidium* does not require dual or multiple hosts in order to undergo a complete developmental cycle. Further, oocysts that are shed do not need special environmental conditions in order to undergo maturation. The oocysts are shed when they are fully sporulated and can survive in the environment for long periods of time. The oocysts are also highly resistant to chlorine and this renders chlorine ineffective as a primary disinfectant used in treatment of drinking water (Chalmers, 2014).

The infective stage for *Cryptosporidium* is the oocysts that are excreted with feces of an infected host such as an infected human or animal. Once ingested, different species of *Cryptosporidium* have different predilection sites in different hosts. For example, *Cryptosporidium parvum* and *C. hominis* infect the small intestines, while *C. muris, C. serpentis*, and *C. andersoni* infect the gastric mucosa (Fayer & Xiao, 2007). In chicken, the species *C. baileyi* prefers the cloaca and the respiratory tree as the primary site of infection. In mice, the cecal junction is the primary site of colonization by *C. parvum*. However, there are reported cases where *Cryptosporidium* has been found in extraintestinal regions in both humans and animal (Fayer & Xiao, 2007).

The sporulated oocyst is the only known exogenous stage. It consists of a tough outer covering that protects and maintains the viability of the 4 sporozoites when under adverse conditions in the environment. The life cycle of *Cryptosporidium* may be divided into six main stages including excystation, merogony, gametogony, fertilization, oocyst wall formation and sporogony (Bouzid *et al* ., 2013). The endogenous phase of the life cycle continues to develop once the oocysts are ingested by a suitable host. Once

ingested, the oocysts undergo excystation where the four infective sporozoites are released from the oocyst through an opening on the oocyst wall (Chen *et al* ., 2002). It is, however, worth noting that suitable environmental conditions are needed for excystation to occur. Specifically, the ingested oocysts need to be exposed to the stomach acids and then to the pancreatic enzymes or bile salts within the small intestines. However, it has been shown that warm aqueous conditions are sufficient to enable excystation (Fayer & Xiao, 2007; Chalmers, 2014). This analogy has been used to account for extra-intestinal infections.

Following excystation, the sporozoites invade host enterocytes at the apical portion of the small intestines and internalize to become the trophozoites (Fayer & Xiao, 2007).The parasites live in a parasitophorous vacuole that is bound by a membrane within host cells. The parasitophorous membrane (PM) protects the parasites from host immune responses and drugs and is the means by which nutrients from the host cells reach the parasite. Trophozoites undergo asexual reproduction (merogony/schizogony/) where the nucleus of the trophozoites divides to form schizonts or meronts (Figure 2.1). For *C. parvum*, the division of the trophozoites results in formation of two types of meronts or schizonts - Type I and type II. Type I meronts develop six to eight nuclei with each of the nuclei being incorporated into a merozoite, while Type II produces four merozoites. Every merozoite leaves a meront to infect the next host cell and then develop into Type I or II meronts (Fayer & Xiao, 2007).

Only Type II meronts initiate sexual reproduction when a new host cell is infected. They either differentiate into a macrogamont (female) or a microgamont (male) stage by a process gametogony (Bouzid *et al* ., 2013). The nucleus of each of the microgamont or microgametocyte undergoes division to form a multinucleated microgamont with each of the nuclei to be incorporated into a microgamete. Macrogamonts, on the other hand, remain uninucleate. For fertilization to occur, the microgamont must attach to and penetrate the cell membrane of the host and the macrogamont. Once inside the

macrogamont, the microgamete either passes its nuclear material to the nuclei of the macrogamont or it enters the nucleus of the macrogamont.

Fertilized macrogamonts give rise to oocysts. The fertilized macrogamonts undergo meiosis to give rise to four sporozoites which develop a trilaminar wall to form oocysts (Fayer & Xiao, 2007; Rossle & Latif, 2013). Oocysts sporulate *in situ* and when they mature they contain four sporozoites by a process sporogony (Caccio & Widmer, 2014). Those oocysts that have thin walls release their sporozoites that auto-infect the host, while those that have thick walls leave the host to infect other hosts (Chen *et al* ., 2002). Oocysts within the gastrointestinal tract leave the host via the feces, while those in the respiratory tract leave the host via secretions of the respiratory system. Oocysts are 3-6µm in diameter with spherical shape and contain four haploid sporozoites (Rossle & Latif, 2013; Caccio & Widmer, 2014).

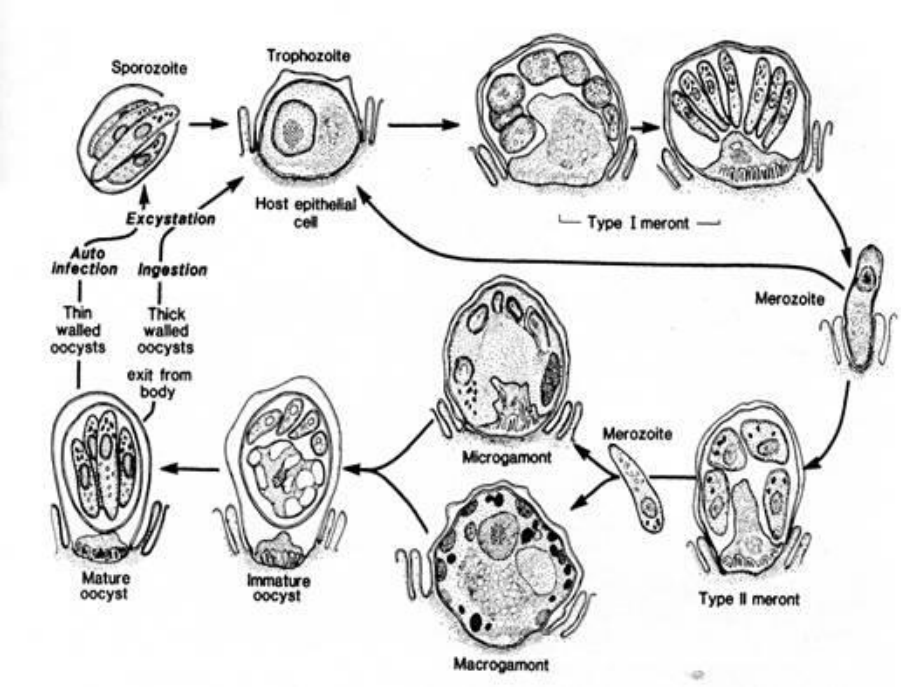

Figure 2.1: Schematic representation of the life cycle of *Cryptosporidium parvum* (Chalmers 2014) .

Fertilized macrogamonts give rise to oocysts. The fertilized macrogamonts undergo meiosis to give rise to four sporozoites which develop a trilaminar wall to form oocysts (Fayer & Xiao, 2007; Rossle & Latif, 2013). Oocysts sporulate *in situ* and when they mature they contain four sporozoites by a process sporogony (Caccio & Widmer, 2014). Those oocysts that have thin walls release their sporozoites that auto-infect the host, while those that have thick walls leave the host to infect other hosts (Chen *et al* ., 2002). Oocysts within the gastrointestinal tract leave the host via the feces, while those in the respiratory tract leave the host via secretions of the respiratory system. Oocysts are 3-6µm in diameter with spherical shape and contain four haploid sporozoites (Rossle & Latif, 2013; Caccio & Widmer, 2014).

2.3 Pathogenesis and clinical symptomatology of *Cryptosporidium* infections

The mechanism by which *Cryptosporidium* causes wasting, diarrhea, and malabsorption are poorly understood. However, the initial interaction between the host and parasite are key elements in pathogenesis of cryptosporidiosis (Wiser, 2012). Multiple complex interactions between several parasite ligands and host receptors during the attachment, invasion and parasitophorous vacuole formation result in development of disease (Singh, 2014). Certain proteins such as the apical complex proteins, namely, CSL (circumsporozoite like antigen), GP900, Gp15, cp 47, cp 60/15, and TRAP C1 (thrombospondin-related adhesive protein of *Cyptosporidium*-1) have been found to mediate complex host-parasite interactions giving rise to the formation of the parasitophorous vacuole, host cell attachment and invasion, and the subsequent development of clinical symptomatology (Tzipori & Ward, 2002; Singh, 2014).

Exposure to pathogenic species of *Cryptosporidium* may result in development of an acute and self-limiting diarrheal disease in immunocompetent persons. Depending on the host and parasite factors, such as the age of the host, previous exposure of the host, the infectious dose, age and origin of the oocysts and the species of the parasite, the onset of the disease may be rapid (3-7 days). Damage to the microvillus border leads to the diminished absorption and digestion. Specifically, there is an alteration of the

cytoskeleton and disruption of the tight junctions resulting in the loss of the barrier functions (Singh, 2014). Diarrhea may last for 7 to 10 days resulting in dehydration and loss of body weight. In cases of reinfection, the disease is less severe.

In immunocompromised individuals such as in HIV and AIDS, infection with *Cryptosporidium* oocysts results in the development of a persistent infection (Mary *et al* ., 2013). Persistent infection is characterized by a profound and life-threatening diarrhea underscoring the significance of this disease in immunocompromised persons where the disease may span several months to years. Existing evidence suggests that there is decreased absorption of sodium and increased secretion of chloride. This increases the osmotic pressure within the intestinal lumen resulting in loss of water with diarrhea as the end result (Wiser, 2012). During this period, the disease may spread to other parts of the gastrointestinal tract such as the pancreatic and hepatobiliary ducts resulting in chalongiohepatitis, choleochitis, pancreatitis, and cholecystitis (Tzipori & Ward, 2002). The mucosal architecture undergoes disorganization characterized by crypt abscessation, cellular infiltrations of the crypt spaces, fibrosis of the intestinal epithelial surfaces.

In the case of malnourished children, the association between chronic disease, presence of persistent diarrhea and malnutrition has not been well established, even though there are reports that *Cryptosporidium*-infected malnourished children are likely to develop persistent diarrhea (Mor *et al* ., 2009). One such study conducted on Ugandan children 3-36 months old, presenting with non-bloody diarrhea, revealed that there was a strong correlation between the occurrence of *C. parvum* and the age distribution of diarrhea (Tzipori & Ward, 2002). The study further revealed that children with persistent diarrhea have a higher prevalence of cryptosporidiosis than children with acute diarrhea; and that children with persistent diarrhea, were more closely associated with stunted growth, underweight, wasted, than those who were healthy (Tzipori & Ward, 2002).

2.4 Diagnosis of cryptosporidiosis

2.4.1 Detection of Cryptosporidium spp. life stages in tissue

Diagnosis of cryptosporidiosis was previously based on the detection of life cycle stages within different samples of human tissue (Fayer & Xiao, 2007). Different developmental stages of *Cryptosporidium* were identified through histology by either biopsy or necropsy of intestinal scrappings. Prepared samples were stained with Haematoxylin and Eosin (H & E) stain before they were examined by microscopy. H & E stained *Cryptosporidium* appeared, under microscopy, as small spherical bodies that measure about 2 to 5µm in diameter (Fayer & Xiao, 2007). For confirmatory diagnosis, Transmission Electron Microscopy (TEM) was used where various features of different life stages of *Cryptosporidium* were visualized. Specimens for biopsies, intestinal scrapings, require the use of invasive procedures to obtain samples for analysis.

The morphology of life cycle stages and variations that exist during development may provide useful information that is species-specific. However, it is notable that examination of different tissues from hosts for morphological identification of the parasite is impractical and this limits the use of biopsies and necropsies for routine diagnosis of cryptosporidiosis. Furthermore, not all intestinal regions may be infected and this can easily give rise to sampling errors. As such, TEM and biopsies/necropsies are no longer used in routine diagnosis of cryptosporidiosis in contemporary diagnostics even though they are still used in investigations on cytoarchitectural and histopathological changes that are closely associated with the infection (Fayer & Xiao, 2007). Better diagnostic techniques such as PCR and LAMP based tests have been developed that can identify and characterize different pathogenic microbes.

2.4.2 Detection of *Cryptosporidium* oocysts

2.4.2.1 Direct smear

Alternative methods to biopsies and necropsies for the detection of various life stages in the diagnosis of cryptosporidiosis exist. These methods rely on the detection of oocysts

in fecal samples. Direct smears can be performed even though they have been found to be particularly insensitive (Casemore *et al.*, 1985). Direct smears may not be useful in detection of *Cryptosporidium* oocysts especially when cysts from other parasites may exist in the same sample or the number of oocysts is low. However, this method is simple to perform and is still used under field conditions in developing countries. Considering low sensitivity of this test, fecal concentration methods have been developed in order to increase its sensitivity.

2.4.2.2 Fecal concentration methods

Fecal concentration methods increase the sensitivity of diagnostic methods such as direct smear by concentrating oocysts from the fecal sample, while reducing debris (Robertson, 2014). However, in patients suffering from acute cryptosporidiosis, there may be no need for fecal concentration because the oocyst number is high. Fecal concentration may be used in specific cases such as in the management of immunocompromised patients who have an unexplained or an uninvestigated previous case of diarrhea (Casemore, 1991). Concentration may also be indicated where it is a requirement for an epidemiological study.

Fecal concentration methods include flotation and sedimentation methods. These methods depend on the differences in specific gravity between the parasite form and the surrounding solution (Baker, 2008). The most commonly used fecal concentration floatation method is the Sheathers sucrose flotation method while the most commonly used sedimentation method is formal-ether concentration method (Vohra, *et al* ., 2012). Sheathers sucrose flotation method is, however, less used because of the fat content in human stool which interferes with its sensitivity. Additionally, Sheathers sucrose floatation method requires that oocysts are thoroughly washed. Otherwise, sucrose could inadvertently interfere with the staining process and adherence of the sample onto the microscope slide.

A number of studies have been conducted to compare the two concentration methods (flotation and sedimentation) in terms of their sensitivity. Results comparing the sensitivity of the two methods are mixed (Vohra *et al*, 2012). According to Cheesbrough (2005), formol-ether concentration method is preferred over Sheathers sucrose floatation because the recovery of oocysts and subsequent staining is better. The World Organization for Animal Health (OIE) recommends a combination of sucrose floatation method with modified Ziehl-Neelson acid-fast staining for the detection of *Cryptosporidium* oocysts from fecal samples (Dhaliwal & Juyal, 2013).

2.4.2.3 Formal ether concentration

This method helps tease the oocysts from other debris such as partly digested food particles that could be present in the sample. Fecal samples are often centrifuged in order to separate the particles in the sample (Fayer & Xiao, 2007). However, because partly digested food particles may sediment and mask the presence of oocysts in microscopic preparations, sieving the samples before centrifugation is recommended. Sieves of multiple pore sizes are used for this purpose. Addition of ether or ethyl acetate to human samples that are fixed or preserved in formalin helps to remove fats and oils that form a fatty plug at the interface of the two liquids after centrifugation (Cheesbrough, 2005). The pellet obtained following centrifugation is retained for examination as the formalin layer, the fatty plug, and the ether layer are discarded.

This method is used routinely for the detection of *Cryptosporidium* oocysts in many clinical laboratories. This method uses acid-fast staining for the detection of the oocysts and this reduces the cost and turn-around time required to perform the test as it is a commonly used technique (Vohra *et al* ., 2012). Further still, this method offers a permanent record of all tests done making it an important diagnostic technique usable in the field where resources may be scarce and repeating tests may be difficult, especially where large numbers of samples are involved. However, this technique is faced with a high level of inter-test variability and even inter-observer variability (Farrar *et al*., 2014). This high test-to-test variability continues to pose a challenge in the diagnosis of

Cryptosporidiosis and creates need for development of more sensitive (microscopy sensitivity is 83.9%), specific, and reliable diagnostic techniques with field applicable properties.

2.4.2.4 Sheather's sucrose floatation method

A recent study comparing the ability of different diagnostic techniques to detect cryptosporidiosis in bovines indicated that sheathers sucrose floatation technique achieved the highest sensitivity levels when compared to other tests. This method was compared to ZN, Kinyuon acid-fast, safranin methylene blue staining, nigrosin staining, light green staining and malachite green staining and found ZN was shown to be the best staining method (Rekha *et al* ., 2016). However, another study comparing routine oocyst purification methods specifically floatation methods such as sodium chloride solution, percolli, and sheathers sucrose solution found that sodium chloride solution achieved the best results. The basis of comparison was the quality of the purification product, the yield or recovery efficacy and reduced the proportion of non-viable oocysts (Kar *et al.*, 2011). When observed under bright field microscopy, the oocysts have a pink tinge that makes it easy to visualize under high power magnification as this does not require skilled manpower. As such, it makes for a good diagnostic technique that can be used routinely in the diagnosis of cryptosporidiosis. However, the wet mounts that are prepared ought to be examined within 15 minutes before the oocysts begin to lose their spherical shape and may collapse (Vohra *et al.*, 2012). This limits the use of this method when large numbers of stool specimen are involved as it may prevent batching of the specimens for diagnostic purposes.

2.4.2.5 Acid-fast staining

Acid-fast stains continue to be routinely used in developing countries in the detection of *Cryptosporidium* oocysts because of their low cost and ease of use. Additionally, acid-fast stains do not need special microscopes in order to detect the oocysts. Their ability to concurrently detect other enteric pathogens such as *Cyclospora* and *Isospora* has

increased its continued use within diagnostic centers in developing countries (Ortega, 2006). Two most widely used staining methods are the modified Ziehl-Neelsen acid-fast staining and the modified Kinyuon's acid-fast staining (Ortega, 2006).

2.4.3 Detection of antibodies/antigens

To detect antigens, antibodies that have been labeled with fluorescent reporters can be used. The antibodies are developed against *Cryptosporidium* oocyst wall antigens. Cp17 and Cp23 are two antigens widely utilized in research for the detection of cryptosporidiosis by Western blot or ELISA. Serological tests based on these two antigens have indicated higher sensitivity and specificity compared to earlier serological tests (Ortega, 2006). Two main types of antigen detection systems that exist include: the enzyme immunoassay assay (EIA) and immunochromatography. Currently, there are commercially available EIA kits in a microwell or microplate format that allow for laboratory testing of large number of samples (Ortega-Pierres, 2009). Current immunochromatographic tests utilize a lateral flow dipstick format in which monoclonal antibodies that are directed at specific membrane proteins within the oocyst wall are used. The test is fast as results are obtained in 5-10 minutes (Sunit, 2014).

Both direct and indirect immunofluorescent methods have been reported to be more sensitive than conventional microscopic methods that use acid-fast staining techniques (Liu, 2014). Specifically, when direct immunofluorescent assay (DFA) was combined with formal-ether concentration, the test achieved a detection limit of 10,000 oocysts per gram of liquid stool and 50,000 oocysts per gram of formed stool compared to 1000 oocysts per gram of liguid stool for. modified Ziehl-Neelsen stain (Liu, 2014). Binding of the antibody paratopes to the antigens on the oocyst surface allows for better visualization of the dimensions of the cyst and further morphometric analysis (Fayer & Xiao, 2007). *Cryptosporidium* oocysts fluoresce to emit characteristic apple-green fluorescence which delineates the oocyst wall, round or slightly ovoid structures that measure 4 to 6µm in diameter.

Enzyme immunoassay (EIA) or enzyme-linked immunosorbent assays (ELISA) are ideal methods that use antibodies conjugated to enzymes to detect antigens (Baveja & Rewari, 2004). Both methods are based on the principle of separating the bound from the unbound and utilize an enzyme as the reporter molecule. These methods do not require fecal concentration in order to obtain the antigen although fecal concentration may help increase sensitivity. In comparison to microscopy, these methods are better as they can offer a more reliable diagnostic outcome in cases where no oocyst has been detected by microscopy (Fayer & Xiao, 2007). Further, they are easy to perform, do not need specialized training of personnel. Despite having higher sensitivity and specificity than microscopy, the suitability of immunodiagnostic tests is easily compromised by antigen variability and the absence of standardized antigens and antibodies for use (Ungar, 1990; Fayer & Xiao, 2007). They also suffer a major limitation; they are unable to differentiate between the *Cryptosporidium* species. Most commercial kits developed for immunological detection of *Cryptosporidium* antigens are based *C. parvum* and *C. hominis* (Liu. 2014). As such, these kits may not be very useful in detecting species of *Cryptosporidium* that are genetically distant from two species. Most EIA commercial kits achieve specificity ranging from 90-100% and a sensitivity ranging from 70-94%. The lower sensitivity levels mean that in patients with lower parasite burdens such as in asymptomatic patients and chronic disease patients, the kits may be unable to detect the parasites (Liu. 2014).

2.4.4 Detection of DNA

With the technological advancements in the medical field, better methods of diagnosis are being developed. These methods are more sensitive, specific, faster, and more reliable. These techniques rely on the detection of certain sections of parasite nucleotide chains/DNA. Detection of DNA as a diagnostic approach is based on a three-step process. The extraction of the DNA, its amplification, and then detection using various detection methods such as gel electrophoresis, SYBR green, autoradiography, and fluorescent dyes (Slack, 2012). There are various methods of DNA amplification that are

employed following DNA extraction and they are broadly divided into three groups. These groups include, target amplification, signal amplification, and probe amplification methods (McClatchey, 2001; Hosler & Murphy, 2014). Target amplification methods include PCR, Nucleic acid sequence based amplification (NASBA), Transcription mediated amplification (TMA), and Strand Displacement Amplification (SDA) (Qian & Lloyd, 2003; Fakruddin *et al* ., 2013). Signal amplification methods include; hybrid capture and branched DNA probes, while probe amplification methods include ligase chain reaction and cleavase invader (Lemon, 2007). A more recent nucleic acid amplification technique is the loop-mediated isothermal amplification (LAMP) (Notomi *et al.*, 2000). Of particular interest in this study are the LAMP and PCR methods.

Both LAMP and PCR rely on the use of primers for the identification of *Cryptosporidium* oocysts both at the genus and species level. To this end, primers play a pivotal role in the determination of the sensitivity and specificity of the test (Fakruddin *et al.*, 2013). The usefulness and effectiveness of primers, in turn, rely on the design process coupled to the gene loci selected. In both cases (PCR and LAMP), primer design is optimized to minimize errors and enable a user to develop the best possible set of primers. Several gene loci have been used to develop primers for detection and identification of *Cryptosporidium* oocysts. Some of the most commonly used genes are SSU 18S rRNA, HSP70, COWP, Actin, and GP60 (Fayer & Xiao, 2007). Sequence diversity exists among species of *Cryptosporidium* over the entire COWP and HSP70, and actin genes making it difficult to use these genes for the design of primers that are genus specific. The 18S SSU rRNA is the best gene locus for use and many PCR-RFLP genus-specific techniques have been described for the differentiation of *Cryptosporidium* species and genotypes (Fayer & Xiao, 2007). The 18S SSU rRNA is more advantageous over other genes because of its high copy number and the presence of conserved regions that have been interspersed within the region in the genome displaying high polymorphism (Sharma *et al* ., 2014). GP60 is a commonly used

subtyping tool for *C. parvum* and *C. hominis* infections in humans and animals (Liu *et al.*, 2015).

2.4.4.1 Polymerase chain reaction (PCR)

Different types of PCR which currently exist include: real-time PCR, reverse transcriptase PCR, broad range PCR, quantitative – competitive PCR, In Situ PCR, Hot Stat PCR, nested PCR, magnetic capture hybridization PCR and their variants such as real-time quantitative reverse transcriptase PCR and multiplex real-time PCR (Yang & Heinsohn, 2007; Harper, 2011; Kumar *et al* ., 2016). For amplification product confirmation, restriction fragment length polymorphism has been widely used digest PCR amplicons (Nichols *et al.*, 2010; Sadek, 2014).

2.4.4.2 Loop-mediated isothermal amplification (LAMP)

Loop-mediated isothermal amplification was developed by Notomi *et al* . (2000) as a novel technique for DNA amplification. LAMP utilizes the autocycling, strand displacement, DNA synthesis activity of the *Bacillus stearothermophilus* (Bst) DNA polymerase together with a set of four to six primers (FIB, BIP, F3, B3) that recognize six to eight distinct sequences on the target DNA strands (Nolan & Bustin, 2013; Abdullahi *et al.*, 2015). LAMP reaction is initiated by the FIP primer that contains sequences of the sense and antisense strands of the target DNA (Notomi *et al* ., 2000). The autocycling reaction results in production of more than 10^9 copies of the target under an hour. A complex set of products is formed that comprises of stem-loop DNAs with several inverted repeats of the target; and cauliflower-like structures with multiple loops formed by annealing between alternate inverted repeats of the gene target in the same strand (Fakruddin *et al* ., 2013; Dhama *et al* ., 2014).

Due to its ability to amplify DNA under isothermal conditions, LAMP enables a user to use simple and cost-effective equipment, such as water bath to amplify DNA and eliminates the need for skilled personnel. Additionally, high amplification efficiency of LAMP makes it possible to amplify DNA without initial heat denaturation of the

template DNA (Njiru, 2012; Abdullahi *et al* ., 2015). As such, the test is able to generate large amounts of DNA over a relatively short period of time, which can be visualized easily using a variety of methods. This greatly reduces the time required for post-amplification analysis. Further, LAMP is able to tolerate most inhibitory compounds that easily affect PCR based assays such as compounds found in cell culture media.

Products of LAMP amplification can be monitored by visualizing the turbidity of the tubes. The tubes can also be pulse centrifuged to deposit the precipitate at the bottom of the tube which is then observed as a white pellet (Mori *et al*., 2001). Turbidometric measurements can also be made to analyze LAMP amplification products (Abdullahi *et al*., 2015). Amplification products can be detected by fluorescence when the pyrophosphate binds to Calcein (Tomita *et al*., 2008; Sahoo *et al*., 2016). Gel electrophoresis can be used to visualize the amplification products by use of fluorescent intercalating dyes, such as ethidium bromide. SYBR green has also been used in to visualize the amplification products where 1µl of SYBR green is added to a single tube of reaction. The change of the Dye's color from orange to green indicates a positive reaction (Monis *et al*., 2005; Parida *et al*., 2005). A ladder-like pattern of the resolved amplicons on agarose gel is visualized under UV (Dhama *et al.*, 2014). Other DNA binding dyes that can be used include Picogreen (Dukes *et al* ., 2006; Curtis *et al*., 2008) and propidium iodide. RFLP and DNA sequencing can be used for amplicon confirmation (Notomi *et al*., 2000; Dhama *et al*., 2014).

LAMP technology has been applied to a wide range of medical fields including clinical diagnosis. Overtime, LAMP has gained wide acceptance as an effective gene amplification tool for use at point of care as a testing device (Njiru, 2011). In regards to *Cryptosporidium,* a number of tests have been developed based on LAMP technology for the detection of *Cryptosporidium* oocyst from both fecal and water samples (Karanis *et al*., 2007; Bakheit *et al*., 2008). Several gene targets such as gp60, COWP, SAM-1, Actin, HSP70, and 18SsRNA genes have been used with18S rRNA being the most commonly used gene target in the development of the tests. The tests have reported

varying degrees of sensitivity and specificity. However, they all note higher sensitivity and specificity of LAMP over PCR based DNA amplification techniques. LAMP has been shown to amplify target DNA from *Cryptosporidium* oocysts up to a third of the samples which previously tested negative for *Cryptosporidium* DNA using nested PCR (Burkheit *et al*., 2008).

2.4.5 Detection and confirmation of amplification products

2.4.5.1 Lateral flow dipstick (LFD)

The test is a simple to use diagnostic device that aids in the detection of certain molecules or target analytes in test samples. They are popular in biomedicine, food, agriculture, and environmental sciences because of their low-cost, simplicity, rapidness, and portability. LFDs are increasingly becoming common in institutions of care because of their ability to provide instantaneous diagnosis at point of care (Koczulla & Gallotta, 2016). They can be qualitative where they are read visually, and quantitative where a reader technology such as ADxLRS is utilized for the quantification of the detected analyte. LFDs are used for the detection of a wide variety of molecules such as antigen, antibodies, and nucleic acids. Moreover, lateral flow immunoassays are increasingly being used at point of care facilities to detect specific molecules in various samples such as plasma, urine, serum, and saliva, among others (Koczulla & Gallotta, 2016). One of the most common lateral flow tests in use today is the pregnancy test.

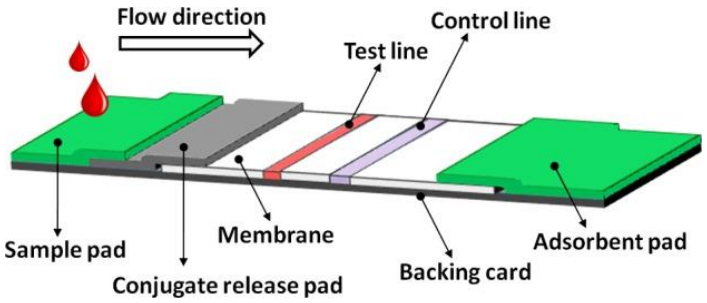

Figure 2.2: Depicts the structure of a lateral flow assay comprising of several elements that assist in the movement of liquids with the target analyte across the membrane for detection (Koczulla & Gallotta, 2016).

LFD tests are based on a simple principle where a liquid containing the analyte or its extract moves across multiple polymeric strips, by capillary action, and can interact with molecules attached on the surface of the strips (Koczulla & Gallotta, 2016). Lateral flow immunoassays consists of four strips namely a sample application pad, conjugate pad, nitrocellulose membrane and adsorption pad (Fig 2.2). The sample application pad is made of glass fibre or cellulose and forms the surface onto which the sample is applied (Sajid *et al.,* 2015). It transports the sample to other parts of the test strip. The conjugate pad contains the labelled bio-recognition molecules which released upon contact with the liquid sample. The nitrocellulose membrane contains the test and control lines where the test results are visualized. All the fluid that flow past the nitrocellulose membrane is collected and held by the adsorbent membrane. In effect, the adsorbent membrane helps ensure that the liquid continuously flows through the test strip.

Lateral flow immunoassays use three main detection formats namely, sandwich, competitive, and multiplex (sajid *et al*., 2015).The current study utilized the sandwich format and will be described briefly. The sandwich version comprises peptide molecules that bind to specific molecules being immobilized on the conjugate pad. A primary peptide against the target analyte is immobilized on the test line while a secondary peptide against labelled antibody conjugate is immobilized on the control line. Once the liquid with the test analyte reaches the conjugate membrane, the labeled peptide combines with the analyte forming the labelled peptide analyte complex. Excess labelled peptide (Sajid *et al*., 2015) solution is captured at the control line and the extra is absorbed by the adsorption pad. The competitive format is based on low molecular weight aptamers that can only bind to one antibody. Multiplex formats have many test lines and can bind to many different molecules such as different species of a test organism. That is, the number of test lines will depend on the number of species under study.

2.4.5.2 Restriction Fragment Length Polymorphism

Restriction enzyme digest is a detection test that was applied in the current study to confirm whether amplification products were *Cryptosporidium* DNA. Enzyme digests only give a positive result if the cleaved DNA is the target DNA. Restriction enzymes are endonucleases that cut double helix DNA at two points (one on each DNA backbone). These points are specific and are known as restriction sites. Some endonucleases may cleave DNA chains at several restriction sites yielding shorter chains of varying sizes. Restriction enzyme digest have been successfully applied widely in the molecular characterization of *Cryptosporidium* species with various endonucleases being used to cleave the DNA at various specific points (Sturbaum *et al* ., 2001; Nichols *et al* ., 2003; Ruecker *et al* ., 2011). In the current study, the NDel endonuclease that cleaves *Cryptosporidium* DNA at position 17 was applied to further prove that the test amplified the right DNA.

2.4.5.3 Gene sequencing

Sequencing is probably one of the most important tools in molecular biology. This tool enables a researcher to determine the exact sequence of nucleotide bases in a DNA strand. Two methods exist the chain termination method and the chemical degradation method (Trietech & Baker, 2013). The chain termination method is more commonly used today because of its ease of use. Sequencing enables users to determine the species and even sub species of target under study. In this study, sequencing was used to determine whether the amplicons by LAMP belonged to *Cryptosporidium* species.

2.5 Treatment, prevention, and control of *Cryptosporidium* infections

The United States Food and Drug Administration (USFDA) recently approved nitazoxanide as the drug of choice in treating diarrhea due to *Cryptosporidium* infections and Giardia (Snelling *et al* ., 2007; Rossle & Latif, 2013). A systematic review of literature had previously assessed assessed the interventions aimed at treating cryptosporidiosis and indicated that there was no effective agent in the management of

cryptosporidiosis among immunocompromised persons (Chen *et al* ., 2002; Abubakar *et al* ., 2007). The effectiveness of nitazoxanide requires a strong immune response (Rossle & Latif, 2013). Among the agents reviewed, the effects of nitazoxanide among HIV seropositive patients were not significant. However, among the HIV seronegative participants, the effect was higher and was associated with a higher relative risk of achieving parasitological clearance (Abubakar *et al* ., 2007). Thus, presently, there is lack of an effective chemotherapeutic agent against cryptosporidiosis among the immune-compromised persons and underscored the significant role of preventive interventions. Supportive therapy i.e fluid and electrolyte replacement, nutritional support and antidiarrhea drugs are vital for the immuno- compromised (Rossle & Latif, 2013).

The use of highly active antiretroviral therapy (HAART) among the immune-compromised persons has resulted in a significant increase in CD4+ T-lymphocyte counts; resulting in an increase in recovery and survival rates (Hunter & Nichols, 2002; Rossle & Latif, 2013). Protease inhibitors which are known to interfere with the life cycle of *Cryptosporidium* parasite, have been combined with Paromomycin, an aminoglycoside, to achieve higher parasite clearance levels among the immune compromised. Further, paromomycin can be combined with recombinant IL-12 to achieve higher parasite clearance efficacy (Rossle & Latif, 2013). Prevention of cryptosporidiosis involves implementing interventions aimed at preventing transmission. Such measures include: washing of raw vegetables thoroughly before consumption, boiling of drinking water, use of clean utensils, and monitoring of water treatment plants for contamination and defective treatment measures. Other measures include quick investigation of outbreaks and isolating the source in order to prevent further transmission; prevent pond water from mixing with municipal water, and train pets to defecate in specific areas to prevent risk of contamination (Rossle & Latif, 2013). Transmission can also occur from livestock; thus, immunocompromised persons should avoid contact with livestock.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study site

This study was based at the Kenya Medical Research Institute's Centre for Microbiology Research (KEMRI-CMR). KEMRI is a national research centre, located within Nairobi County and engages in human research involving a wide array of infectious and non-infectious diseases. The Centre for Microbiology Research receives its samples from Mbagathi hospital (a government hospital which is closely located to KEMRI) and Reuben Centre and Medical Missionaries of Mary clinics located in informal settlements such as Mukuru Kwa Njenga and Mukuru Reuben. CMR also receives samples from other surrounding hospitals and clinics within its environs.

3.2 Study design

This study adopted a quantitative approach with a cross-sectional study design. The study processes were organized as shown in Figure 3.1.

3.3 Ethical approval

The study was approved by the Scientific Steering Committee (SSC) (Appendix 1) and the Ethical Review Committee (ERC) (Appendix 2) now Scientific Ethics Review Committee of the Kenya Medical Research Institute (SERU) (SSC No. 2891) before the commencement of the study. The study used archived DNA samples from a previous study SSC number 1579 by Mbae *et al*. (2013) whose objectives were to genotype and serotype *Cryptosporidium* spp. Samples were analyzed anonymously. As such, no personal identifying information was used in the study or publication arising out of it. Each sample was identified by a unique study number.

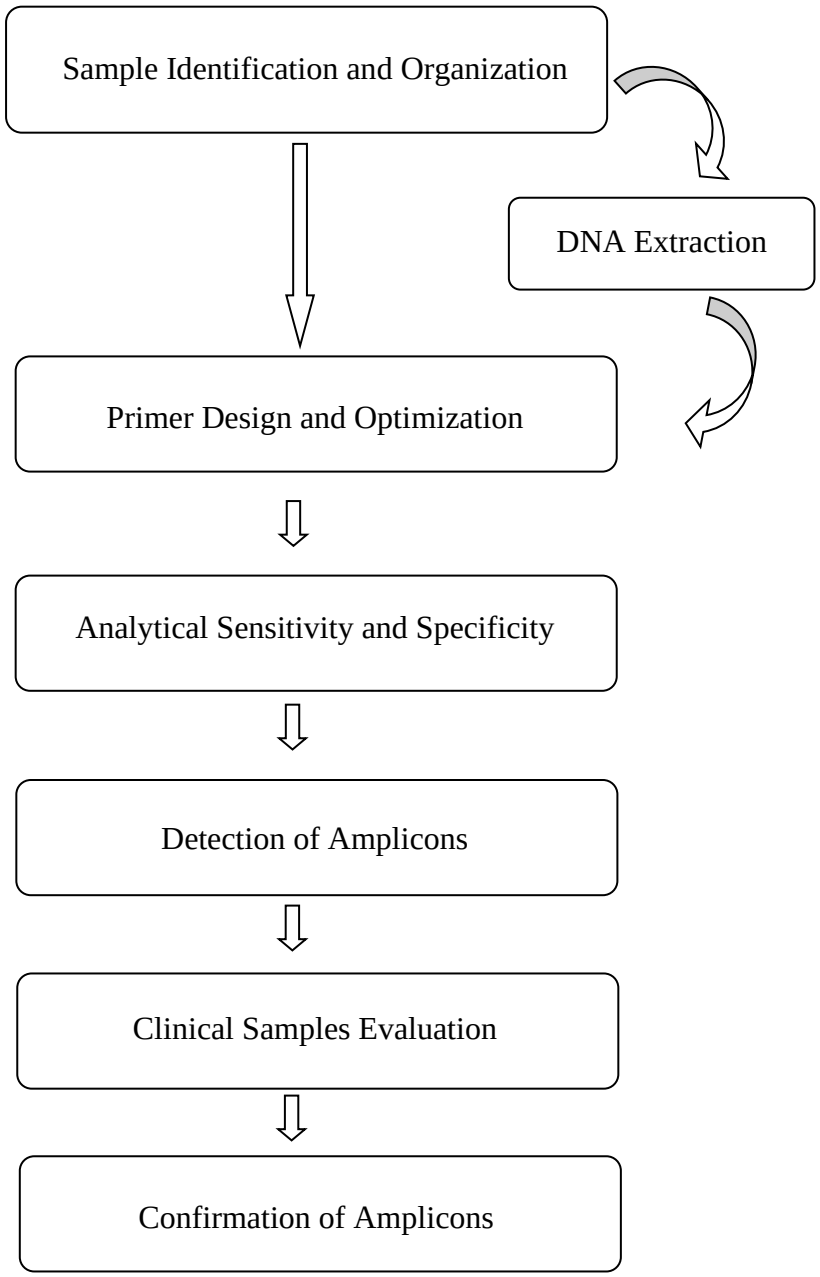

Figure 3.1: Schematic representation of the study design and experimental setup. Archived samples were used. Confirmation was achieved by sequencing and detection by LFD, Gel electrophoresis, and SYBR green.

3.4 Sample size estimation

According to a recent study, the prevalence of cryptosporidiosis in Kenyan children younger than 15 year presenting with or without diarrhea ranged from 3.7% to 9.8% (Squire & Una, 2017). This study, therefore, used the average prevalence obtained as follows:

$$[a + b]/2 \text{ where}$$

(a) is the lower range (3.7%) and (b) is the higher limit(9.8%)

$$[3.7 + 9.8]/2$$

$$= 6.75\% = 0.0675$$

The sample size for the study was obtained using a described by Cochran (1963). Notably, this formula has been recommended for use in cross-sectional types of studies (Charan & Biswas, 2013). The formula is as follows

$$n = Z^2 \times p\, q / d^2$$

Where n = Desired sample size (target population > 10,000)

Z = Standard normal deviation at the required confidence interval (1.96)

p = proportion estimated to have measured character in the target population (0.0675) obtained above

$$q = 1 - p$$

d = level of statistical significance at 95% confidence level = 0.05

The calculations are as follows;

$$= (1.96)^2 \times 0.0675 \times (1-0.0675)/.05^2$$

$$=3.8416 \times 0.0675 \times 0.933/0.0025$$

$$=3.8416 \times 0.0675 \times 373.2$$

$$= 97$$

3.5 Sample identification and organization

All the archived DNA samples (n-106) were obtained and arranged based on the unique identifying code in an ascending order. Out of the 106 DNA samples used (Table 2), 39 had been previously confirmed to be *Cryptosporidium* positive by gene sequencing and later archived (Mbae et al., 2013). The remaining 67 samples comprised of DNA clinical samples that were not sequenced but were also archived. Each of the samples, in liquid form, was examined for quantity by visual inspection. About 20µl of DNA per sample was required, and those samples with low volume were identified and earmarked for extraction. The source of specimen, sample collection, host type, transportation and storage are not described in the current study, considering that archived samples were used. However, these procedures are described in detail in Mbae et al. (2013).

3.6 DNA extraction

Genomic DNA was extracted from the fecal specimen preserved in 2.5% potassium dichromate using QiAmp® DNA Stool Mini kit (Qiagen, United Kingdom) as per the manufacturer instructions with slight modifications. Briefly, 200 µl of the fecal suspension was washed five times with triple-distilled water by centrifugation. To this suspension, 1.4 ml of ASL buffer was added and subjected to five times thawing (80°C) and freezing (-80°C) to rupture the rigid oocysts. The genomic DNA was eluted in 50 µl of nuclease-free water and stored at -20°C until use.

3.7 LAMP primer design

3.7.1 Primer design

To design the primers, three species of *Cryptosporidium* commonly found in Kenya were used including *C. parvum*, *C. hominis,* and *C. meleagridis*. The primers were designed based on the three genes from the three species. Thus, three sequences, for each *Cryptosporidium* spp. were obtained from gene bank rather than one specific SAM-

1 *C. parvum* sequence (cgd7_2650) from cryptodb database. Their nucleotide sequences were obtained from Genbank using their accession numbers (Table 3.1).

Table 3.1: showing the Genbank accession numbers for the SAM-1 gene

***Cryptosporidium* Species**	**Genbank accession number**
C. parvum	AB119646.1
C. hominis	XM_662396
C. meleagridis	AB119648

Each of the nucleotide sequences was arranged without spaces, given that the GeneBank output usually has spaces between nucleotide sequences (Figure 3.2) and was then copied and pasted onto the box in step 1 of the online Clustal Omega programme (**http://www.ebi.ac.uk/Tools/msa/clustalo/**). At step 2 of the programme, the clustalW numbers format was selected and then submitted. The main purpose of the Clustal Omega programme was to achieve multiple sequence alignment.

Using the product of multiple sequence alignment (Figure 3.3) the regions with the highest alignment (all three bases align) were selected for primer design. Note – on the multiple sequence alignment product means that two bases align at that point, * means that three bases align at that point and a blank space means that the bases differ at that point. Regular primers, the outer and inner primers were designed using primer explorer version 4 (https://primerexplorer.jp/e/) and the selection of the primers was based on a criterion provided by Notomi *et al*. (2000). The primers were designed based on the region showing the highest homology on the multiple sequence alignment product (Figure 3.4).

>AB119646.1 parvum

aatagacgag tcatctctga ataactagtc ttggtattat taaacggctg gaagctaatc
ttttgcatac gcggtattat tttcttaata ctcgaaaaga taaatcattt cttgtgcaga
ttcgtaaata ataatatatt tgttaaatct tttattgtgt taaatagtta tggattcttc
gaggcttagt ggaaataaat ccacttacac tgacttacaa actacttctg agcaattttt
attttcttca gagtcagttt gtagtggcca cccagataaa ttatgtgatc agatttcgga
tgcaatcctt gatgcgtgct tggaacaaga tccagaaagc tttgtagcgt gtgaaacatg
cacaaaaaca gggttcatta tggtttttgg tgaaataact acaaaggcta atgtaaatta
cgaaagagtt gtaagagaaa cagtgaaaga aattggatat gactctgaag aaaaagggtt
agattacaaa actatggacg tgattattaa gctagaacaa caaagtaatc aaattgctgg
gtgtgtacat gtagataaaa atgtagaaga tattggagcg ggtgatcaag gaatgatgtt
tggttatgct acgaatgaaa caaaagaact catgcctctg acgcacgtat tagctacatc
tattacaaga gagctggatt atatcagaat gaaaggagta tcttctcggg tgggttggct
gcgccctgat ggaaaggcgc aagtgacagt agaatataac tgcaaacatg gcgtactcat
tccaaaaaga attcacacta ttttagtttc ggttcaacat gatgaaaaca tagaaaacga
ggaaattaga gaatttgttc tggagaatgt aattaaaaaa gtatgccctt cagatttgat
ggacaaagaa actagaatat taattaatcc atctggcaga tttacaattg gggggccagc
agcagatgct ggattaacag ggcgcaagat aattgtagat acatacggag gatggggtgc
tcatgggggt ggtgcattta gcgggaaaga tgcaactaaa gtagatagat caggtgcata
tatggcaaga cttgttgcaa agtcaatcgt cttttctggc ttgtgtagca gatgtttggt
acaggtttca tatggaattg gaatagcaag gcctttatca ctatatatta atacatttgg
cacagcgaaa gatgggtata atgacacaaa actactggag atagttaata aggtatttga
ttttaggcca ggaatcttaa ttaagcagct aaatcttaaa tctcctattt ttaaaaagac
atcaagtggc ggacattttg gacgatcaga aaaagagttt ctttgggaaa agccaattat
tttacaatag aataatattg taaacattta gtcattatat actaaaatcg attttaggcg
gccttcattt tttttgttaa acctgactaa ttaaattata attctattt

>XM_662396 hominis

atggattctt cgaggcttag tggaaataaa tccacttaca ctgacttaca aactacttct
gagcaatttt tattttcttc agagtcagtt tgtagtggcc acccagataa attatgtgat
cagatttcgg atgcaatcct tgatgcgtgc ttggaacaag atccagaaag ctttgtagcg
tgtgaaacat gcacaaaaac agggttcatt atggtttttg gtgaaataac tacaaaggct
aatgtaaatt acgaaagagt tgtaagagaa acagtgaaag aaattggata tgactctgaa
gaaaaagggt tagattacaa aactatggac gtgattatta agctagaaca acaaagtaat
caaattgctg ggtgtgtaca tgtagataaa aatgtagaag atattggagc gggtgatcaa
ggaatgatgt ttggttatgc tacgaatgaa acaaaagaac tcatgcctct gacgcacgta
ttagctacat ctattacaag agagctggat tatatcagaa tgaaaggagt atcttctcgg
gtgggttggc tgcgccctga tggaaaggcg caagtgacag tagaatataa ctgcaaacat
ggcgtactca ttccaaaaag aattcacact attttagttt cggttcaaca tgatgaaaac
atagaaaacg aggaaattag agaatttgtt ctggagaatg taattaaaaa agtatgccct
tcagatttga tggacaaaga aactagaata ttaattaatc catctggcag atttacaatt
ggggggccag cagcagatgc tggattaaca gggcgcaaga taattgtaga tacatacgga
ggatggggtg ctcatggggg tggtgcattt agcgggaaag atgcaactaa agtagataga
tcaggtgcat atatggcaag acttgttgca aagtcaatcg tcttttctgg cttgtgtagc
agatgtttgg tacaggtttc atatggaatt ggaatagcaa ggcctttatc actatatatt
aatacatttg gcacagcgaa agatgggtat aatgacacaa aactactgga gatagttaat
aaggtatttg attttaggcc aggaatctta attaagcagc taaatcttaa atctcctatt
tttaaaaaga catcaagtgg cggacatttt ggacgatcag aaaaagagtt tctttgggaa
aagccaatta ttttacaata g

>AB119648 meleagridis

aatagatgat tattctgaat aactagtttg tattattaaa cgattgcgaa taatttttgt
atacgcggtt ttgtttcctt aatactcgaa agataaatta tttcttgtgc agattagtaa
ataactatat atttgttaaa ttttgatcgt gttaaatggt tatggattct ttgaggctta

```
gtggaaataa acccacttac actgacttac aaactgcttc tgaacaattt ttattttctt
cagagtctgt atgtagtggc cacccagata aattatgtga tcagatttcg gatgcaattc
ttgatgcgtg cttggagcaa gatccagaaa gctttgttgc atgtgaaaca tgcacaaaaa
cagggttcat tatggttttt ggtgaaataa ctacaaaggc taatgtgaat tacgaaagag
ttgtgagaga aacagtgaaa gaaataggat atgactctga agaaaaaggg ttggattaca
aaactatgga tgtgattatt aagctagaac aacaaagcaa tcaaattgct ggctgtgtac
atgtaaataa aaatgtagaa gatattggag cgggtgatca aggaatgatg tttggctatg
ctacgaatga aacaaaagaa ctcatgcctc taacgcacgt attagctaca tctatcacaa
gagagctgga ttatattaga atgaaagaag catcttctcg ggtgggttgg ctacgtcctg
atggaaaggc gcaagtgaca gtagaataca actgcaagca cggagtactc attccaaaga
gaattcacac tattttagtt tcggttcaac atgatgaaaa catagaaaac gagaaaatta
gagaatttgt tctggaaaat gtgattaaaa aagtatgccc ttcagatttg atagacaaag
aaactagaat attaattaat ccatctggca gatttacaat tgggggggcca gcagcggatg
ctggattaac agggcgcaaa ataattgtag atacatacgg aggatggggt gcacatggag
gtggtgcatt tagcgggaaa gatgcaacta aagtagatag gtcaggcgca tatatggcaa
ggcttgttgc aaagtcaatc gtcttttctg gattgtgtag cagatgtttg gtgcaggttt
catatggaat tggaatagca aagcctttat cactgtatat taatacattt ggcacagcga
aagatgggta taatgacaca aaactgctgg agatagttaa taaggtgttt gattttaggc
```

Fig 3.2: showing the nucleotide sequences of the three *Cryptosporidium* species obtained from the GenBank database using their respective accession numbers.

```
AB119648        aatagat--gattattctgaataacta--gtttgtattattaaacgattg--cgaataat
AB119646.1      aatagacgagtcatctctgaataactagtcttggtattattaaacggctggaagctaatc
XM_662396       ------------------------------------------------------------

AB119648        ttttgtatacgcggttttgtttccttaatact-cgaaagataaattatttcttgtgcaga
AB119646.1      ttttgcatacgcggtattattttcttaatactcgaaaagataaatcatttcttgtgcaga
XM_662396       ------------------------------------------------------------

AB119648        ttagtaaataactatatatttgttaaa-ttttgatcgtgttaaatggttatggattcttt
AB119646.1      ttcgtaaataataatatatttgttaaatcttttattgtgttaaatagttatggattcttc
XM_662396       -----------------------------------------------atggattcttc
                                                               **********
AB119648        gaggcttagtggaaataaacccacttacactgacttacaaactgcttctgaacaattttt
AB119646.1      gaggcttagtggaaataaatccacttacactgacttacaaactacttctgagcaattttt
XM_662396       gaggcttagtggaaataaatccacttacactgacttacaaactacttctgagcaattttt
                ******************* ********************** ******* ********
AB119648        attttcttcagagtctgtatgtagtggccacccagataaattatgtgatcagatttcgga
AB119646.1      attttcttcagagtcagtttgtagtggccacccagataaattatgtgatcagatttcgga
XM_662396       attttcttcagagtcagtttgtagtggccacccagataaattatgtgatcagatttcgga
                *************** ** ****************************************
AB119648        tgcaattcttgatgcgtgcttggagcaagatccagaaagctttgttgcatgtgaaacatg
AB119646.1      tgcaatccttgatgcgtgcttggaacaagatccagaaagctttgtagcgtgtgaaacatg
XM_662396       tgcaatccttgatgcgtgcttggaacaagatccagaaagctttgtagcgtgtgaaacatg
                ****** ***************** ******************** ** ***********
AB119648        cacaaaaacagggttcattatggtttttggtgaaataactacaaaggctaatgtgaatta
AB119646.1      cacaaaaacagggttcattatggtttttggtgaaataactacaaaggctaatgtaaatta
XM_662396       cacaaaaacagggttcattatggtttttggtgaaataactacaaaggctaatgtaaatta
                ************************************************** *****
AB119648        cgaaagagttgtgagagaaacagtgaaagaaataggatatgactctgaagaaaaagggtt
AB119646.1      cgaaagagttgtaagagaaacagtgaaagaaattggatatgactctgaagaaaaagggtt
XM_662396       cgaaagagttgtaagagaaacagtgaaagaaattggatatgactctgaagaaaaagggtt
```

```
                ************ ******************** *************************
AB119648        ggattacaaaactatggatgtgattattaagctagaacaacaaagcaatcaaattgctgg
AB119646.1      agattacaaaactatggacgtgattattaagctagaacaacaaagtaatcaaattgctgg
XM_662396       agattacaaaactatggacgtgattattaagctagaacaacaaagtaatcaaattgctgg
                 ***************** ************************* **************
AB119648        ctgtgtacatgtaaataaaaatgtagaagatattggagcgggtgatcaaggaatgatgtt
AB119646.1      gtgtgtacatgtagataaaaatgtagaagatattggagcgggtgatcaaggaatgatgtt
XM_662396       gtgtgtacatgtagataaaaatgtagaagatattggagcgggtgatcaaggaatgatgtt
                 ************ ********************************************
AB119648        tggctatgctacgaatgaaacaaaagaactcatgcctctaacgcacgtattagctacatc
AB119646.1      tggttatgctacgaatgaaacaaaagaactcatgcctctgacgcacgtattagctacatc
XM_662396       tggttatgctacgaatgaaacaaaagaactcatgcctctgacgcacgtattagctacatc
                *** ********************************** ********************
AB119648        tatcacaagagagctggattatattagaatgaaagaagcatcttctcgggtgggttggct
AB119646.1      tattacaagagagctggattatatcagaatgaaaggagtatcttctcgggtgggttggct
XM_662396       tattacaagagagctggattatatcagaatgaaaggagtatcttctcgggtgggttggct
                *** ******************** ********** ** **********************
```

Figure 3.3: Multiple sequence alignment of the three nucleotide sequences retrieved from GenBank

The loop and stem primers were manually designed using the formulae $T_m = 4(G+C) + 2(A+T)$. Notably, the T_m for F1c and B1c was set at 64° while that of the rest of the primers was set between 58-60°. The F1c and B1c are complementary forward primer 1 and complementary backward primer 1 representing specific regions on the target DNA. The development of a LFD format differentiated this test from previous designs.

```
AB119648    agcggatgctggattaacagggcgcaaaataattgtagatacatacggaggatggggtgc
AB119646.1  agcagatgctggattaacagggcgcaagataattgtagatacatacggaggatggggtgc
XM_662396   agcagatgctggattaacagggcgcaagataattgtagatacatacggaggatggggtgc
            *** ********************** ********************************

AB119648    acatggaggtggtgcatttagcgggaaagatgcaactaaagtagataggtcaggcgcata
AB119646.1  tcatgggggtggtgcatttagcgggaaagatgcaactaaagtagatagatcaggtgcata
XM_662396   tcatgggggtggtgcatttagcgggaaagatgcaactaaagtagatagatcaggtgcata
             ***** ****************************************** ***** *****

AB119648    tatggcaaggcttgttgcaaagtcaatcgtcttttctggattgtgtagcagatgtttggt
AB119646.1  tatggcaagacttgttgcaaagtcaatcgtcttttctggcttgtgtagcagatgtttggt
XM_662396   tatggcaagacttgttgcaaagtcaatcgtcttttctggcttgtgtagcagatgtttggt
            ********* ***************************** ********************

AB119648    gcaggtttcatatggaattggaatagcaaagcctttatcactgtatattaatacatttgg
AB119646.1  acaggtttcatatggaattggaatagcaaggcctttatcactatatattaatacatttgg
XM_662396   acaggtttcatatggaattggaatagcaaggcctttatcactatatattaatacatttgg
             *************************** ************ *****************

AB119648    cacagcgaaagatgggtataatgacacaaaactgctggagatagttaataaggtgtttga
AB119646.1  cacagcgaaagatgggtataatgacacaaaactactggagatagttaataaggtatttga
XM_662396   cacagcgaaagatgggtataatgacacaaaactactggagatagttaataaggtatttga
```

```
           ********************************* ******************** *****

AB119648   ttttaggccaggaatcttaattaagcagctaaaccttaaatctcctatattcaaaaagac
AB119646.1 ttttaggccaggaatcttaattaagcagctaaatcttaaatctcctatttttaaaaagac
XM_662396  ttttaggccaggaatcttaattaagcagctaaatcttaaatctcctatttttaaaaagac

           *********************************
```

Figure 3.4: Regions within the multiple alignment product showing the highest homology selected for manual primer design.

The regions showing the highest homology were selected as indicated and the nucleotide sequence from F3 and B3 highlighted. F3 and B3 are forward and backward outer primers on the target gene sequence. The nucleotide sequence from F3 to B3 was blasted using the basic local alignment search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for target specificity. The nucleotide sequence was blasted to ensure that the final set of primers were specific for the three species being investigated. The F3B3 sequence was then converted to a double-stranded sequence (http://www.bioinformatics.org/sms/rev_comp.html) and all the primers mapped out (Figure 3.5). Mapping out was done to identify the annealing position of the primers and also for purposes of identifying the RFLP cutting site. The F3-B3 sequence with the highlighted primers, was copied from the multiple sequence alignment product to obtain a single sequence which was blasted to confirm 100% identity (Figure 3.6).

```
AB119648   agcggatgctggattaacagggcgcaaaataattgtagatacatacggaggatggggtgc
AB119646.1 agcagatgctggattaacagggcgcaagataattgtagatacatacggaggatggggtgc
XM_662396  agcagatgctggattaacagggcgcaagataattgtagatacatacggaggatggggtgc
           *** ********************** *******************************

AB119648   acatggaggtggtgcatttagcgggaaagatgcaactaaagtagataggtcaggcgcata
AB119646.1 tcatggggggtggtgcatttagcgggaaagatgcaactaaagtagatagatcaggtgcata
XM_662396  tcatgggggtggtgcatttagcgggaaagatgcaactaaagtagatagatcaggtgcata
            ***** ***************************************** ***** *****

AB119648   tatggcaaggcttgttgcaaagtcaatcgtcttttctggattgtgtagcagatgtttggt
AB119646.1 tatggcaagacttgttgcaaagtcaatcgtcttttctggcttgtgtagcagatgtttggt
XM_662396  tatggcaagacttgttgcaaagtcaatcgtcttttctggcttgtgtagcagatgtttggt
           ********* ***************************** ********************

AB119648   gcaggtttcatatggaattggaatagcaaagcctttatcactgtatattaatacatttgg
AB119646.1 acaggtttcatatggaattggaatagcaaggcctttatcactatatattaatacatttgg
XM_662396  acaggtttcatatggaattggaatagcaaggcctttatcactatatattaatacatttgg
            *************************** ************ ****************
```

```
AB119648    cacagcgaaagatgggtataatgacacaaaactgctggagatagttaataaggtgtttga
AB119646.1  cacagcgaaagatgggtataatgacacaaaactactggagatagttaataaggtatttga
XM_662396   cacagcgaaagatgggtataatgacacaaaactactggagatagttaataaggtatttga
            ******************************** ******************** *****

AB119648    ttttaggccaggaatcttaattaagcagctaaaccttaaatctcctatattcaaaaagac
AB119646.1  ttttaggccaggaatcttaattaagcagctaaatcttaaatctcctatttttaaaaagac
XM_662396   ttttaggccaggaatcttaattaagcagctaaatcttaaatctcctatttttaaaaagac

            ********************************
```

Figure 3.5: Regions with the highest homology on the multiple sequence alignment with all the primer sequences mapped out.

Gaggatggggtgctcatgggggtggtgcatttagcgggaaagatgcaactaaagtagatagatcaggtgcatatatggcaag acttgttgcaaagtcaatcgtcttttctggcttgtgtagcagatgtttggtacaggtttcatatggaattggaatagcaaggcctttat cactatatattaatacatttggcacagcgaaagatgggtataatgacacaaaactactggagatagttaataaggtatttgattttag gccaggaatcttaattaagc

Figure 3.6: Nucleotide sequence indicating the location of the primers for the stem LFD LAMP test.

The final primers are presented in Table 3.2 below.

Table 3.2: showing the final primers for the stem SAM-1 LAMP test

Primer	Base pairs	Sequence	T_m(°C)
F3	18	GAGGATGGGGTGCTCATG	(11 ×4)(7×2) = 58
B3	22	CCTTATTAACTATCTCCAGYAG	(8×4)(14×2) = 60
F1C	22	GACTTTGCAACAAGTCTTGCCA	(10×4)(12×2) = 64
F2	20	GCATTTAGCGGGAAAGATGC	(10×4)(10×2) = 60
FIP	42	GACTTTGCAACAAGYCTTGCCA-GCATTTAGCGGGAAAGATGC	
LF	21	CRCCTGAYCTATCTACTTTAG	(8×4)(13×2) = 58
STEM F	21	TACACAAKCCAGAAAAGACGA	(9×4)(12×2)= 60
STEM R	22	TGTTTGGTRCAGGTTTCATATG	(8×4)(14×2) = 60
B1C	23	ATTGGAATAGCAAGGCCTTTATC	(9×4)(14×2) = 64
B2		GTCATTATACCCATCTTTCGC	(9×4)(12×2) = 60
BIP	44	ATTGGAATAGCAARGCCTTTATC-GTCATTATACCCATCTTTCGC	
LB	23	CTRTATATTAATACATTTGGCAC	(6×4)(17×2)= 58

3.7.2 Lateral flow primer design

To design the lateral flow, the forward inner primer (FIP) was labeled with biotin in the 5' end. A probe to detect biotinylated LAMP product was designed between primers B1c and F1c. the probe was then labeled using fluorescein isothiocyanate (FITC).

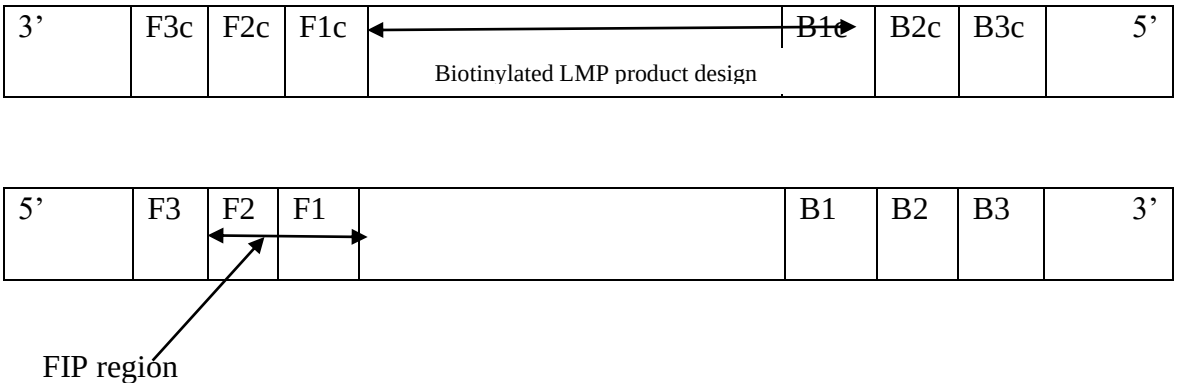

Figure 3.7: Schematic representation of the design and labeling of lateral dipstick probe.

3.8. Primer optimization- Taguchi method

The Taguchi method enables users to determine the effects and interactions of different reaction components using few reactions (Cobb & Clarkson, 1994). For instance, a test investigating the effects and interactions of four reaction components, each at three levels of concentration, would require a total of 81 reactions (3^4). However, with the application of Taguchi methods, only 9 reactions are required to determine the effects and interactions of the test. Provided that the number of concentrations per reaction component is three, then the total number of experiments required to test for the effects and interactions of each reaction component is given by the equation E = 2K + 1 where k is the number of reaction components to be tested. In this study, the primers were optimized using the modified Taguchi method as described by Cobb and Clarkson (1994). Briefly FIP/BIP were varied from 30-60pmoles, stem primers at 10-30pmoles, loop primers at 10-30pmoles, dNTPs 1-3mM, reaction temperatures 63-65▫C, and

displacement primers 3-5mM. The new experimental test was labeled stem SAM-1 LAMP test.

3.9 Analytical sensitivity and specificity

Ten-fold serial dilutions of *Cryptosporidium* control DNA were conducted in duplicates under standard LAMP conditions (Notomi *et al*., 2000) in order to establish the analytical sensitivity of the standard LAMP assay, the stem SAM-1 LAMP test, and the PCR test. The LAMP primers were analyzed in the following combinations: [i] With outer primers - F3/B3, FIP/BIP, LF/LB SF/SB and [ii] without outer primers -FIP/BIP, LF/LB, SF/SB. To check whether the stem LFD LAMP format analytical sensitivity could be improved further, preheated templates were used. Briefly, the LAMP master mix was divided into 25 µl reaction tubes and placed in the incubation chamber at ~63^{o}C. After approximately 3 minutes, 2µl of the preheated template (genomic DNA) was added to each respective tube and reactions left to run for 60 minutes. To check time to results for different LAMP formats, a dilution of 10^{-4} (~1000 oocysts/ml) of reference *C. hominis* DNA was used, and reactions ran for 25, 30, 35, 40, and 45 minutes. For each time schedule, the reaction tubes were transferred to a thermal block set at 80^{o}C to stop the reaction. For SAM-1 LAMP and nested PCR, the expected products were analyzed using 2% agarose gel. The stem LFD LAMP test specificity was checked using *Toxoplasma gondii, Giardia duodenalis, Entamoeba histolytica, Ascaris lumbricoides, Cyclospora species* and human DNA. These parasites were selected because their DNA is closely related to that of cryptosporidium. It was necessary to ensure that the test does not give false positive was tested on samples with these parasites. They were obtained from archived DNA and tests were run as described for *Cryptosporidium.*

3.10 Evaluation of clinical samples

3.10.1 LAMP

Archived clinical samples (n=67) were evaluated as follows. LAMP reactions for the stem SAM-1 LAMP test and the standard LAMP assay were conducted under standard

LAMP conditions. Briefly, 25µl LAMP reactions comprising of 2.5 µl 10X reaction buffer, 2mM dNTPs, 40pmols of FIP/BIP, 20pmols of LF/LB, 20pmol SF/SB, 1M betaine and 8000U/ml Bst polymerase per reaction. The LAMP reactions were conducted using a PCR thermocycler (Applied Biosystems, CA, USA) set at 63°C for a period of an hour. LAMP reactions were duplicated using a water bath as the source of heat. The template was 2µl of genomic DNA. All clinical samples were analyzed in duplicates and repeated once after two weeks. For the current study, the final LAMP product included the sequence from F2 to B2. As such, the outer primers, F3 and B3 did not form part of the bands observed under UV on the enzyme digest agarose gel. The final nucleotide sequence excluding the outer primers was 220bp long in the form of a ladder-like patterns.

3.10.2 PCR

All the clinical samples (n=67) were subjected to nested PCR as described by Alves *et al* (2003) with a few modifications. The expected size of the PCR product was approximately 800bp. Briefly, 25 µl reactions comprising of 2.5 µl of 10X reaction buffer, 2mM dNTPs, 10 µm of Alves F1, Alves R2 and 20mM of Taq polymerase. The PCR reactions were conducted as follows; 35 cycles of 94˚C for one minute, 55˚C for 30 seconds and 72˚C for 30 seconds. The PCR underwent a 94˚C for 4-minute at the beginning of cycling and a 72˚C 7-min period at the end of cycling, and amplicons were stored at 4˚C. A nested PCR was also conducted the initial amplification was done using forward primer (5'-ATAGTCTCCGCTGTATTC-3') and reverse primer (5'-GAGATATATCTTGGTGCG-3'). After which 2µl of the PCR product was used in the second round of reactions. The secondary PCR used a forward primer (5'-TCCGCTGTATTCTCAGCC-3') and reverse primer (5'-GCAGAGGAACCAGCATC-3'). The amplification conditions were as described above with the addition of 2mM $MgCl_2$.

3.10 Detection of PCR and LAMP amplification products

3.10.1 Gel electrophoresis and SYBR ® Green I Dye

The amplicons, both LAMP and PCR were visualized under 2% agarose gels stained with 10µl of ethidium bromide. Products of both LAMP assays were also detected by SYBR ® Green 1 dye as per the manufacturer's instructions.

3.10.2 Lateral flow dipstick

After the LAMP reaction, the LFD hybridization was performed by incubating LAMP products with 20 pmol of FITC labeled probe at 63 °C for 5 min in a final volume of 20 µl followed by the addition of 8µl of the reaction mixture and 150 µl of the reaction assay buffer. The LFD strip (Millennia® HybriDetect, Millennia Biotec, Germany) were then dipped into the mixture for 5 min at room temperature. The test was considered positive when both the control and test lines appeared.

3.10.3 Restriction enzyme digest

A restriction map was drawn using NEBCutter V2 - http://nc2.neb.com/NEBcutter2/ online programme based on the nucleotide sequence from F2 to B2, excluding the outer primers. The map gives an indication of the enzymes that can digest the LAMP product and size of the resulting digest products. Based on the restriction map obtained the NDel enzyme was selected. The Ndel enzyme cuts at one point, resulting in two bands on the gel making it the most suitable enzyme for this study. The enzyme cuts at position 117 resulting in two bands, one 117bp, and the other 103bp.

The enzyme digest was conducted as follows; 2µl of each LAMP amplification product was digested using the Ndel restriction enzyme (New England Biolabs, MA, USA) as per the manufacturer's instructions with a few modifications. Briefly, 5µl of 1X NEBuffer was mixed with 42µl of nuclease-free water, 1µl of DNA and 2µl of the restriction enzyme to make a total volume of 50 µl. The endonuclease reaction was incubated overnight. The restriction enzyme digest products were detected by 2% agarose gels stained with 10µl of ethidium bromide and visualized under UV light.

3.11 Confirmation of the LAMP products

3.11.1 Gene sequencing

Confirmation of the amplification products was achieved by sequencing, which was done in Australia. The uppermost LAMP amplicon was excised from the agarose gel, cloned, transformed and inserts sequenced using an automated DNA 3730 analyzer. The resulting sequences were aligned with the target sequences using the DNAman computer software (Lynnon, USA).

3.12 Data analysis

3.12.1 Descriptive statistics

The samples (n =106) were divided into two groups comprising the already confirmed positive samples (by sequencing) (n=39) and the clinical samples (n=67). The number of positive samples (in each group, n = 39 and n = 67) was calculated as a percentage of the total samples in each of the two groups for each of the three tests. For instance, the percentage of positive samples detected by PCR (p) for the confirmed positive samples was calculated as follows

$$[p/39]*100$$

where p is the number of positive samples detected by PCR.

The process was repeated for the clinical samples (n =67) for each of the three tests. The samples were separated into two groups so that a better estimate for percentage agreement can be obtained using confirmed samples. However, this statistical test was also performed for all the samples combined (n=106).

3.12.2 Percentage agreement

Interrater reliability (IR) is an important concept in healthcare practice. Determining the level of agreement between various diagnostics tests helps increase the level of confidence in the results presented (McHugh, 2012). The higher the interrater reliability the higher the confidence in the study results and the higher the accuracy of the results.

Percentage agreement is one of the methods used to measure interrater reliability. One of the benefits of this method is that it is simple to perform. Additionally, the method allows a researcher to identify whether the errors are randomly distributed or one of the raters consistently makes different judgements from the other raters (McHugh, 2012). A researcher can also identify problematic variables.

To obtain the interrater reliability of the three tests, a matrix was created in which the columns represented the three tests and the rows represented the measured variables (the values were either a positive result or negative result). The percentage agreement was calculated for each row and the mean (IR) for all the row averages were obtained for each of the two groups (n=39, and n = 67). See illustration below using five samples. The five samples used below are for illustration purposes and do not represent actual results. The results of this calculation are presented in the next chapter.

Table 3.3: Table illustrating how percentage agreement was obtained using random sample results

Samples	**Raters**			
	Sam-1 LAMP	**LFD Stem SAM-1 LAMP**	**PCR**	**% Agreement**
1	+	+	-	66
2	+	+	+	100
3	+	-	-	66
4	-	+	-	66
5	+	+	+	100
IR (Average of the total % agreement)				79.6

3.12.3 Cohen's Kappa test

The Kappa (k) statistic is another method of measuring interrater reliability. Similar to a correlation coefficient, it can range from -1 to +1 where 1 represents perfect agreement and values below 0 represent disagreement. When the values are below zero, in a clinical setting it is necessary to retrain raters or redesign the instruments. A kappa value > or equal to 64% is desirable (McHugh, 2012). However, values below 35% indicate inadequate agreement between raters can such results can only elicit little confidence.

In this study the following ranges were used for the interpretation of the study results.

Table 3.4: Showing the kappa results interpretation scheme

Kappa Value	Agreement level	Percentage reliability (%)
0 – 0.20	None	0 - 4
0.21 - 0.39	Minimal	4 -15
0.40 - 0.59	Weak	15 - 55
0.60 – 0.79	Moderate	35 - 63
0.80 – 0.90	Strong	64 - 81
Above 0.90	Almost perfect	82 – 100

Kappa was calculated using the formula by McHugh (2012)

$$K = \frac{OA-EA}{1-EA}$$

Where (OA) is the observed agreement and (EA) is the expected agreement. Notably, Stem SAM- 1 LAMP test was compared to SAM – 1 LAMP test and PCR and results presented in a 2 by 2 matrix from where the Kappa statistic was derived.

3.13 Data management

All samples that were used in this study had unique codes that were assigned to them as they were received at the clinic in Mukuru kwa Njenga. Further, all data that was generated was entered in a laboratory book and MS excel worksheets for storage. The data storage locations were only accessible to the study investigators.

CHAPTER FOUR

4.0 RESULTS

4.1 Optimization of LAMP primers

The modified Taguchi method was used to optimize the primers and the results indicated in Table 4.1 below.

Table 4.1: The final optimized concentrations of the primers as determined by the modified Taguchi method. The primers have been described in the methods section.

Primer	**F3**	**B3**	**FIP**	**BIP**	**LF**	**LB**	**SF**	**SR**
Conc. (Pmoles)	5	5	40	40	20	20	20	20

4.2 Analytical sensitivity and specificity

4.2.1 Sensitivity

4.2.1.1 Stem LFD SAM-1 LAMP

Ten-fold serial dilutions of *C. parvum* and *C. hominis* reference DNA were used to determine the analytical sensitivity of the stem LFD SAM-1 LAMP test in the following combinations; with outer primers and without the outer primers. The analytical sensitivity in both cases was 10 oocysts/ml. Notably, an average of 2 of 6 replicates in every run, or approximately 30% of the replicates consistently showed detection limit of ~1 oocysts/ml when the template was preheated. Time to results from master mix preparation was 80 minutes for the stem LFD LAMP using gel electrophoresis.

4.2.1.2 SAM-1 LAMP test

Ten-fold serial dilutions of the SAM-1 LAMP test indicated that the test had an analytical sensitivity of 100 oocysts/ml (Table 4.2). Time to results from master mix preparation was 120 minutes using gel electrophoresis.

4.2.1.3 Nested PCR

Ten-fold serial dilution of the nested PCR test indicated an analytical sensitivity of 100 oocysts/ ml (Table 4.2). Time to results was 320 minutes using gel electrophoresis.

4.2.2 Specificity

Closely related DNA was used to determine the specificity of the test. The DNA included *Toxoplasma gondii, Giardia duodenalis, Entamoeba histolytica, Ascaris lumbricoides,* and *Cyclospora species.* The stem SAM-1 LAMP test indicated 100% specificity – no amplified product was seen (Table 4.2).

Table 4.2: The analytical sensitivities of the LAMP and PCR tests and specificity of the stem LFD LAMP test
[a] the result of the test was the same with or without the use of outer primers, which means outer primers have no effect on the results and can be ommitted
[b]30% of replicates indicated an analytical sensitivity of 1 oocyst/ml on heating the template for Stem LFD

Test	Analytical Sensitivity (oocysts/ml)	Specificity (%)
Stem LFD LAMP[a]	10	100
Preheated template[b]	1	100
SAM-1 LAMP test	100	100
Nested PCR	100	100

4.3 Descriptive statistics

4.3.1 LFD Stem SAM-1 LAMP test

The stem LFD LAMP assay was able to detect 29/39 sequenced (Mbae et al., 2013) samples and 16/67 (23.8%) clinical samples. The time to results from master mix preparation to gel electrophoresis was 80 minutes. Notably, loop and stem primers were used in this test (Table 4.3).

Table 4.3: Evaluation of confirmed *Cryptosporidium* spp. DNA samples and clinical samples
[a] SAM-1 LAMP test
[b] SSU rRNA nested PCR
[c] Template was pre-heated for 5 minutes to check its effect on sensitivity
[d] Detection using gel electrophoresis
[f] From master mix preparation to visual result readout
[†] The samples were also positive using stem LFD LAMP test
Nd: not done

		Types of Test		
	Indices	**Stem LFD LAMP**	**SAM-1 LAMP[a]**	**Nested PCR[b]**
***Cryptosporidium* spp DNA (N = 39)**				
	No. Positive	29(74.4%)	27(69.2%)	25(64.1%)
Clinical samples (N = 67)	No. Positive	16(23.9%); 19(28.4%)[c]	14(20.8%)[†]	11(16.4%)[†]
	Time to results (Min)[f]	80	120[d]	320[d]
	Accelerating primers	Loop and stem	Loop	nd

4.3.2 SAM-1 LAMP test

The SAM-1 LAMP test detected 27/39 (69.2%) sequenced samples and 14/67 (20.9%) clinical samples. The time to results from master mix preparation to gel electrophoresis was 80 minutes. Notably, only loop primers were used in this test (Table 4.3).

4.3.3 Nested PCR

The nested PCR test detected 25/39(64.1%) sequenced samples and 11/67 (16.41%) clinical samples. The time to results from master mix preparation to gel electrophoresis was 320 minutes (Table 4.3).

4.4 Percentage agreement

The interrater reliability of the three tests was conducted and found to be 77.22% the study samples (n = 106). The IR for the confirmed samples (n =39) was 76.46% and for the clinical samples (n = 67) the IR was 77.67%.

4.5 Kappa statistic

The kappa statistic was determined using the 2 by 2 matrix shown below. The results of the kappa statistic are presented below and are as follows: LFD Stem Sam-1 LAMP versus SAM-1 LAMP (n=106)(Table 4.4); LDF Stem SAM-1 LAMP versus PCR (n=106) (Table 4.5); LFD Stem SAM-1 LAMP versus SAM-1 LAMP (n=39) (Table 4.6); LFD Stem SAM-1 LAMP versus PCR (n=29)(Table 4.7).

Table 4.4: 2 by 2 matrix comparing Stem LFD stem SAM-1 LAMP to SAM-1 LAMP for the kappa statistic (n =106)

	Stem LFD SAM-1 LAMP			
		Positive	Negative	Total
SAM-1 LAMP	Positive	39	16	55
	Negative	26	25	51
	Total	65	41	106

Given that

$$K = \frac{OA-EA}{1-EA}$$

$$OA = (39+25)/106 = 0.603$$

$$EA = (65/106)\times(55/106) + (41/106) \times (51/106) = (0.613\times0.518) + (0.387\times0.481) = 0.504$$

$$K = 0.22$$

Where EA is expected agreement

Table 4.5: 2 by 2 matrix comparing LFD Stem SAM-1 LAMP to PCR for the kappa statistic (n =106)

	Stem LFD SAM-1 LAMP			
		Positive	Negative	Total
PCR	Positive	31	18	49
	Negative	36	21	57
	Total	67	39	106

$$OA = (31 + 21)/106 = 0.\ 491$$

$$EA = (67/106) \times (49/106) + (39/106) \times (57/106) = (0.632 \times 0.462) + (0.368 \times 0.538) = 0.489$$

$$K = 0.004$$

Table 4.6: 2 by 2 matrix comparing LFD Stem SAM-1 LAMP to SAM-1 LAMP for the kappa statistic (n =39)

	Stem LFD SAM-1 LAMP			
		Positive	Negative	Total
SAM-1 LAMP	Positive	12	6	18
	Negative	10	11	21
	Total	22	17	39

$$OA = (12 + 11)/39 = 0.59$$

$$EA = (22/39) \times (18/39) + (17/39) \times (21/39) = (0.564) \times (0.461) + (0.436) \times (0.538) = 0.49$$

$$K = 0.16$$

Table 4.7: 2 by 2 matrix comparing LFD Stem SAM-1 LAMP to PCR for the kappa statistic (n = 39)

	Stem LFD SAM-1 LAMP			
		Positive	Negative	Total
PCR	Positive	8	8	16
	Negative	14	9	23
	Total	22	17	39

$$OA = (8 + 9)/39 = 0.436$$

$$EA = (22/39) \times (19/39) + (17/39) \times (23/39) = (0.564) \times (0.487) + (0.436) \times (0.590) = 0.532$$

$$K = -0.2$$

The low Kappa values indicate low agreement between the test and the negative kappa test means there was no agreement. The low agreement could be attributed to the fact that archived DNA samples had been used and could have degenerated by the time of the study was conducted. Fresh samples could yield better results.

4.6 Detection of LAMP and PCR products

4.6.1 Gel electrophoresis

The amplification products for the LAMP tests and PCR were detected using 2% agarose gels stained with ethidium bromide. The positive stem LAMP products showed the ladder-like pattern on the agarose gel indicating the formation of stem-loops with inverted repeats (Figure 4.1) while the PCR agarose gels indicated the characteristic bands (Figure 4.2).

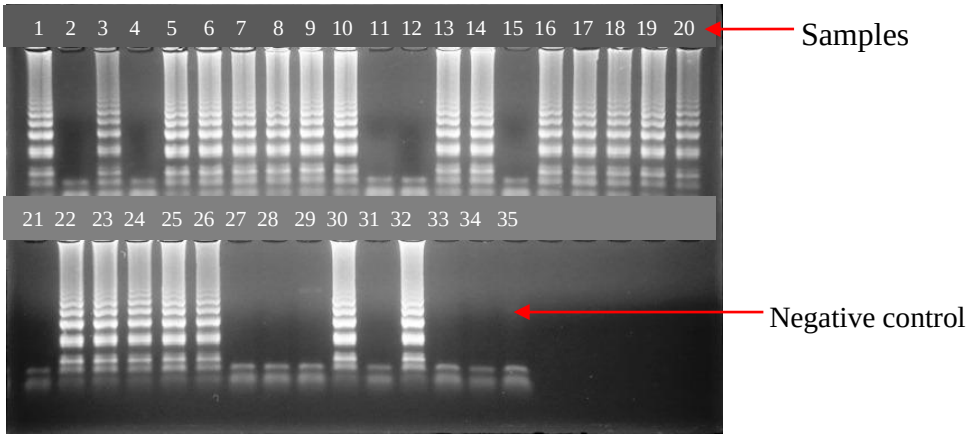

Figure 4.1: The characteristic ladder-like pattern displayed by clinical samples that tested positive by LAMP. Sample 2, 4,11, 12, 15, 21, 27, 29, 31, 33, and 34 were negative samples while the rest were positive samples. Lane 35 was the negative control.

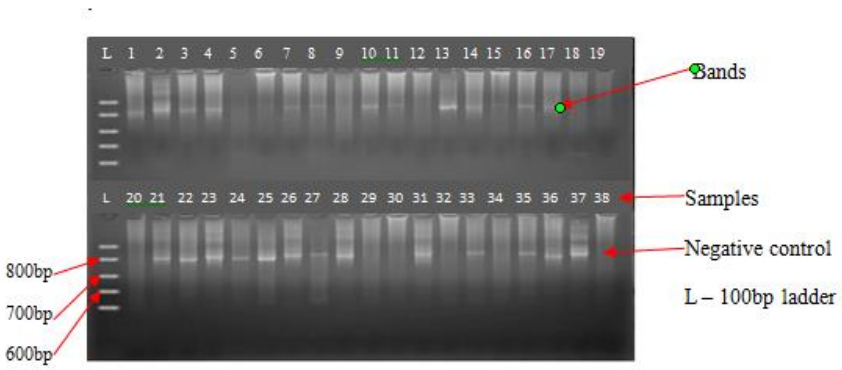

Figure 4.2: Gel electrophoresis results of the nested PCR amplification products. Lanes 1-37 were samples while lane 38 was the negative control. A 100bp DNA ladder was used and the bands are approximately 800bp (Thermo Fisher, MA, USA).

4.6.2 SYBR®Green I dye

The LAMP amplification products were also detected using the SYBR®Green I Dye. Products that were positive by LAMP had a green coloration while the negative clinical samples had an orange coloration (Figure 4.4A & B).

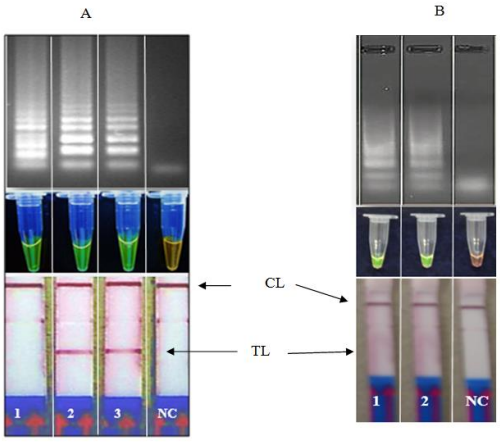

Figure 4.3: **(A)** The detection of stem LAMP product from some selected reactions done for over 60 minutes using 2.0% agarose gels stained with ethidium bromide, SYBR Green I dye and LFD dipstick format. 1= (False positive), 2 = *C. hominis*, 3 = *C. parvum* and NC= PCR water. **(B)** The appearance of non-specific products at 75 minutes reaction cut-off time. 1, M044 (False positive), 2, M099 (False positive), NC = PCR water. TL = test line, CL = control line. The non-specific products show different patterns agarose gel and turn green on the addition of SYBR Green I. However, none was positive using the LFD format. The false positives could be due to formation of primer dimmers.

4.6.3 Lateral Flow dipstick format

The positive stem LAMP products showed the ladder-like pattern on the agarose gel indicating the formation of stem-loops with inverted repeats (Figure 4.4a) and the expected test line on the LFD strip (Figure 4.4b).

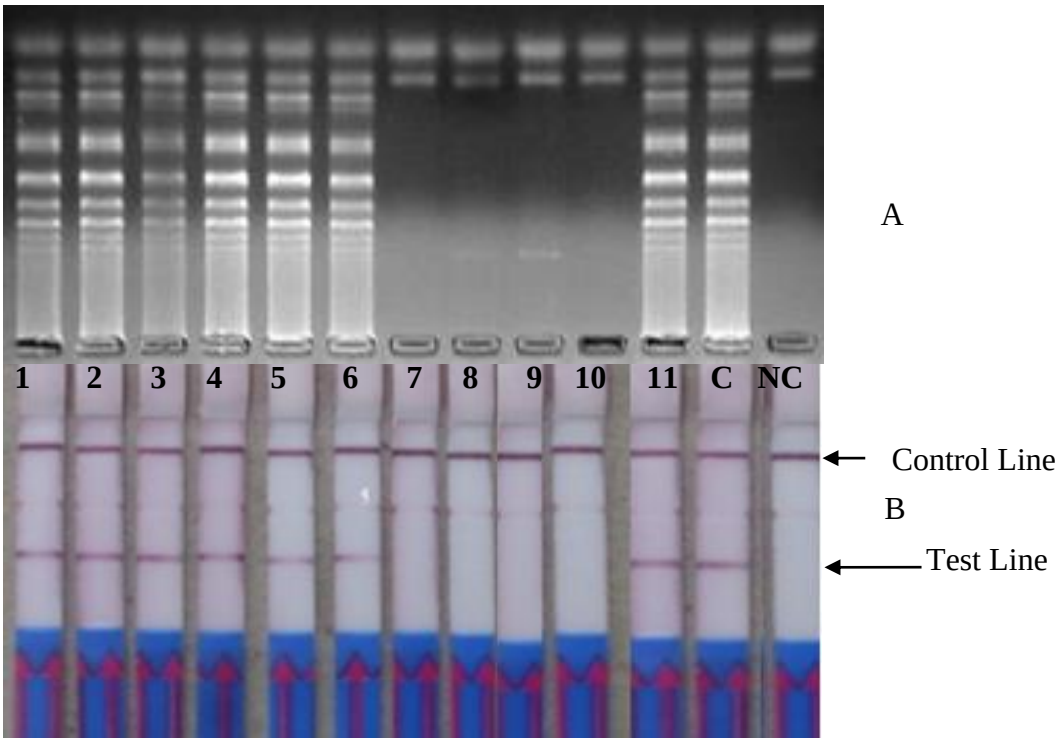

Figure 4.4: (A) The detection *Cryptosporidium* spp using the stem LAMP amplification product (220bp) using 2.0% agarose gel stained with ethidium bromide. **(B)** LFD results. The faint line between the test line and the positive control line is nonspecific binding at DIG test line because the strips were done to detect two products. 1 =MB407, 2 = MB419, 3 = MB491, 4 = MB501, 5 = MB502, 6 = M1492, 7= M1599, 8 = M009, 9 = M016, 10 = M044, 11= M074, C= *C. hominis* DNA and NC= PCR water. The M and MB are sample lablels.

4.6.4 Restriction enzyme digest

The restriction enzyme digestion of the stem LAMP amplicons indicated the predicted amplicons of approximately 117 bp and 103 bp (Fig 4.5). No restriction enzyme digestion was recorded from samples with inconsistent banding patterns.

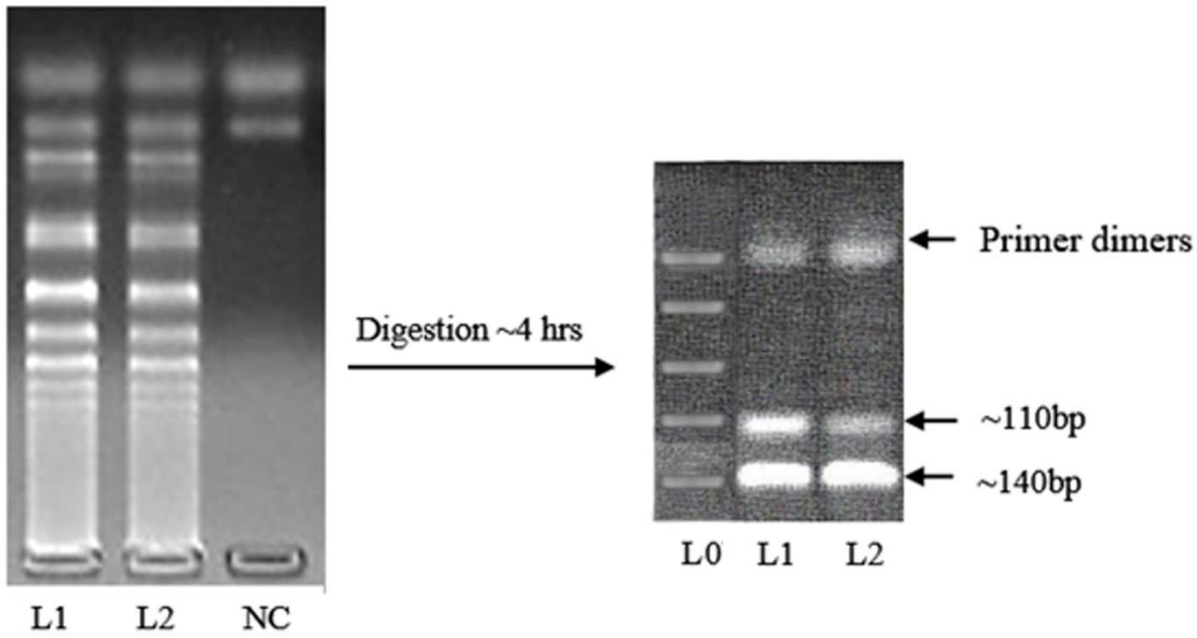

Fig 4.5: Gel electrophoresis of stem LAMP product for *C. hominis* (L1) and *C. parvum* (L2), Ladder (L0)) and their *Nde*I restriction enzyme digest (highly magnified). The predicted amplicon sizes based on the *Nde*I cutting site is ~104 bp (80bp + primer B1 24bp) and ~137bp (115 bp + primer F1c 22 bp). NC = negative. The results of the enzyme digest are in agreement with the estimated products after the digest. One product of the digest was approximately 104bp while the other was roughly 137 bp. Thesse two products are in agreement with the enzyme digest products: one which is approximately110 and 140 bp respectively.

4.7 Confirmation of LAMP products

4.7.1Gene sequencing

The sequence from the uppermost amplicon of randomly selected stem LAMP positive samples indicated high sequence homology with SAM gene sequences from *C. hominis*, *C. parvum.*

```
M13.16          Gactttgcaacaagacttgccagcatttagcgggaaagatgcaactaaagtagatagatc          60
M12.64          Gactttgcaacaagacttgccagcatttagcgggaaagatgcaactaaagtagatagatc          60
M14.28          Gactttgcaacaagacttgccagcatttagcgggaaagatgcaactaaagtagatagatc          60
M12.111         Gactttgcaacaagacttgccagcatttagcgggaaagatgcaactaaagtagatagatc          60
AB119648        atggggtgcacatggaggtggtgcatttagcgggaaagatgcaactaaagtagataggtc          60
AB119646.1      atggggtgctcatgggggtggtgcatttagcgggaaagatgcaactaaagtagatagatc          60
XM_662396       atggggtgctcatgggggtggtgcatttagcgggaaagatgcaactaaagtagatagatc          60
                          ** *        ********************************** **

M13.16          aggtgcatatatggcaagacttgttgcaaagtcaatcgtcttttctggcttgtgtagcag          120
M12.64          aggtgcatatatggcaagacttgttgcaaagtcaatcgtcttttctg-gttgtgtagcag          119
M14.28          aggtgcatatatggcaagacttgttgcaaagtcaatcgtcttttctggcttgtgtagcag          120
M12.111         aggtgcatatatggcaagacttgttgcaaagtcaatcgtcttttctggcttgtgtagcag          120
AB119648        aggcgcatatatggcaaggcttgttgcaaagtcaatcgtcttttctggattgtgtagcag          120
AB119646.1      aggtgcatatatggcaagacttgttgcaaagtcaatcgtcttttctggcttgtgtagcag          120
XM_662396       aggtgcatatatggcaagacttgttgcaaagtcaatcgtcttttctggcttgtgtagcag          120
                *** ************** ***************************  ************

M13.16          atgtttggta--ggtttcatatggaattggaatagcaaggcctttatcactatatattaa          178
M12.64          atgtttggtacaggtttcatatggaattggaatagcaaggcctttatcactatatattaa          179
M14.28          atgtttggtacaggtttcatatggaattggaatagcaaggcctttatcactatttattaa          180
M12.111         atgtttggt-caggtttcatatggaattggaatagcaaggcctttatcactatttattaa          179
AB119648        atgtttggtgcaggtttcatatggaattggaatagcaaagcctttatcactgtatattaa          180
AB119646.1      atgtttggtacaggtttcatatggaattggaatagcaaggcctttatcactatatattaa          180
XM_662396       atgtttggtacaggtttcatatggaattggaatagcaaggcctttatcactatatattaa          180
                *********   ************************** ************ * ******

M13.16          tacatttggcacagcgaaagatg          201
M12.64          tacatttggcacagcgaaagatg          202
M14.28          tacatttggcacagcgaaagatg          203
M12.111         tacatttggcacagcgaaagatg          202
AB119648        tacatttggcacagcgaaagatg          203
AB119646.1      tacatttggcacagcgaaagatg          203
XM_662396       tacatttggcacagcgaaagatg          203
                ***********************
```

Figure 4.6: The multiple alignment of ~200 bp sequence sections obtained after sequencing the uppermost amplicon from four samples that were positive with stem LAMP test but negative using other comparative tests. The F1c (boxed) and F2 (shaded grey) form FIP primer. A partial B2c sequence (shaded black) is part of BIP primer. The sequence shows >96% identity with the target. sequence AB119646.1 for *C. parvum*, XM662396.1 for *C. hominis* and AB119648.1 for *C. meleagridis*. NB. The sequences obtained will differ depending on the band sequenced and whether the sequence was initiated by FIP or BIP primers.

Another major aim of the study was to assess the applicability of the LAMP test to field conditions and compare it to PCR. Table 4.8 below compares the benefits of using LAMP to Microscopy – which is the main diagnostic method used in field conditions in developing countries. In addition, the current study compared the use of PCR to the current LAMP test based on cost (Table 4.9).

Table 4.8: A comparison of LAMP based assay with microscopy in field conditions

Attribute	**LAMP**	**Microscopy**
Batch processing	Yes	No
Time spent	I hour per run of 96 samples	Fewer samples per hour
Sensitivity	74% (current study)	64% (current study)
Test-to-test variability	low	High
Skill level	low	high

Table 4.9: A cost analysis comparing the cost of conducting a LAMP test to PCR test in field conditions. The prices for the machines may vary from place to place. Nonetheless, it is significantly lower for LAMP test. The prices of the machines are from Lab X (an online platform https://www.labx.com/product/laboratory-water-bath) and PCR machine (Labome https://www.labome.com/method/PCR-Machines.html)

Input	LFD Stem SAM-1 LAMP (Kshs)	PCR (Kshs)
Cost of Machine	Water bath 40,000	Machine 259,500
Labour	Similar – process is same	Similar
Electricity	Lower–shorter time (60 minutes per run)	Higher (320 minutes for nested PCR – current study results)
Reagents	Slightly higher – uses more reagents, such as primers	Lower – uses fewer reagents

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

The main aim of this study was to develop a modified lateral flow dipstick format Loop-mediated isothermal amplification (LAMP) test for detection of *C. parvum, C. hominis* and *C. meleagridis* in stool samples of patients. Cryptosporidiosis is a disease affecting over 450 million people globally with immunocompromised persons such as in HIV and AIDS being the worst affected (Ortbald *et al.*, 2013). Notably, in developing countries, particularly sub-Saharan Africa, up to 50 percent of deaths among HIV and AIDS infected persons are attributable to *Cryptosporidium* spp. infections (Ortbald *et al.*, 2013). Considering the disease burden and the lack of affordable field applicable sensitive diagnostic tests, it was prudent to develop a sensitive and specific LAMP based test that can aid in the diagnosis of *Cryptosporidium* infections. Current diagnosis in field conditions depends on microscopy which suffers low sensitivity (83.7%). Loop-mediated isothermal amplification of DNA is a method that has gained momentum in the diagnosis of different micro-organisms due to its inherent advantages of high sensitivity, specificity and its potential applicability in resource-poor endemic areas (Notomi *et al* . 2015). A LAMP based Cryptosporidium test was developed to *detect C. parvum, C. hominis,* and *C. meleagridis* but achieved low sensitivity at 33.4% (Bakheit et al., 2008). The low sensitivity created a need for better diagnostic tests. The current study successfully used a second set of reaction accelerating primers (stem primers) combined with a lateral flow dipstick format to design a sensitive LAMP test capable of detecting *C. hominis, C. parvum* and *C. meleagridis* based on the SAM-1 gene. To this end, this study presents the development and evaluation of a sensitive and specific LAMP based test for the detection of *Cryptosporidium* oocysts.

The current study presents the development and evaluation of a LAMP based test based on the SAM-1 gene target. The sensitivity and specificity of a new test are based on the target gene used. Ideally, a gene with high copy numbers is preferable for primer design. In past PCR and LAMP based *Cryptosporidium* detection tests, several genes have been identified and used for the design of primers. In the case of PCR based tests, some of the genes used include GP60 (Alves *et al*., 2003), SSU rRNA (Xiao *et al* ., 1999; Limor *et al* ., 2002; Coupe, *et al*., 2005), and HSP70 (Sulaiman, *et al*., 2000). More recently, GP60 (Christen *et al* ., 2014), 18S rRNA (Iqbal, 2012; Stensvold *et al*., 2015), and SSU rRNA (Hadfield *et al*., 2011) have been used for the development of PCR based *Cryptosporidium* oocysts detection tests. Given that LAMP is a more recent technology, fewer LAMP based tests for the detection of *Cryptosporidium* oocysts have been developed (Karanis *et al*., 2007; Bakheit *et al*., 2008; Koloren, 2017). One of the studies compared several gene targets and concluded that SAM-1 gene was the most suitable for primer design (Bakheit *et al*., 2008). In this regard, the SAM-1 gene was regarded the most suitable gene targets for this study. The combined use of primer explorer version 4 with optimal primer design conditions for the displacement primers (F3, B3) and loop generating primers (FIP, BIP) as described (Notomi *et al* ., 2000) has been shown to yield high-quality LAMP primers. However, the design of an additional set of reaction accelerating primers – the stem primers and the loop primers were designed manually. Several recent LAMP based studies have successfully utilized primer explorer version 4 for primer design (Arunrut *et al* ., 2016; Martzy *et al* ., 2017; Njiru *et al* ., 2017).

The analytical sensitivity of the stem SAM-1 LAMP test was conducted in two formats with and without outer primers. Recently, the ratio of outer and inner primers in LAMP studies has been an issue of interest as it is widely held that the ratio of inner to outer primers concentrations directly affects the sensitivity of a test. In this regard, several studies have been conducted to determine the most appropriate ratio applicable to LAMP based tests. Findings from several studies indicate that the use of ratios with higher concentrations of inner primers yield tests that enjoy higher sensitivity levels

(Cao *et al*., 2014; Su *et al*., 2016). This study established that there was no difference in terms of sensitivity between the two formats implying that the use of outer primers had no significant effect on the sensitivity of the test. The omission of outer F3 and B3 primers in this stem LAMP format indicated poor test performance confirming that outer primers have varied effects on different stem LAMP tests (Gandleman *et al*., 2011). For this reason, subsequent tests were conducted with the exclusion of outer primers. Future studies can be conducted without outer primers, given that they have no effect on sensitivity yet they may increase formation of primer dimmers.

A comparison of the analytical sensitivity of the stem LFD LAMP, SAM-1 LAMP tests, and nested PCR revealed that there were significant differences. Stem LFD LAMP test was able to achieve high analytical sensitivity levels of 10oocysts/ml compared to 100 oocysts/ml of the SAM-1 LAMP and nested PCR tests. Moreover, the stem LFD LAMP test indicated a shorter time to results comparatively. The higher analytical sensitivity levels and shorter time to results recorded by the stem LFD LAMP test could be attributed to the use of reaction accelerating primers (loop and stem primers) that generated large amounts of amplification product compared to the SAM-1 LAMP format which relies only on loop primers. The loop primers accelerate the reaction by priming the sequence loops (Nagamine *et al*., 2002) while the stem primers accelerate the reaction by targeting the stem section of the sequence (Gandleman *et al*., 2011). Thus, this set of primers could be employed in future studies to boost the sensitivity of LAMP tests. The use of preheated template marginally improved the stem LFD LAMP test sensitivity by 10-fold. A possible explanation for the higher sensitivity levels achieved with preheating the template could be that pre-heating unwinds target DNA hence provides more target for priming and/or heating to accelerate betaine destabilization of the target DNA bonds subsequently making it easier to displace the outer primers.

The higher analytical sensitivity and better comparative analysis of the stem LFD LAMP test translated into higher detection rates on clinical samples analysis when compared to nested PCR and SAM-1 LAMP tests. Further, the higher detection limits recorded by the

stem LFD LAMP test due to pre-heating the template translated to a higher detection rate of pathogen DNA by ~4.6 percent from clinical specimen when compared to SAM-1 LAMP test (Table 4.3). The higher detection of rates of *Cryptosporidium* DNA from clinical samples compared to SSU rRNA nested PCR agrees with previous results (Bakheit *et al*., 2008; Gallas *et al*., 2016).

The study compared the three tests (PCR, stem LFD SAM -1 LAMP and SAM-1 LAMP) in order to determine the interrater reliability. The percentage agreement test revealed that there was a high level of agreement between the tests (77.22 where n =106, 76.46 where n = 39 and 77.67 where n = 67). However, the percentage agreement test fails to factor in the level of agreement that can be attributed to chance (McHugh, 2012). In this regard, the Kappa test was performed in order to determine the level of agreement between the tests after adjusting for chance agreement. Based on the study findings, the level of agreement between the stem LFD SAM-1 LAMP test and the SAM-LAMP test was minimal (k = 0.22 where n = 106 and k =0.16 n = 0.16). However, there was no agreement between Stem LFD SAM-1 LAMP and PCR (k – 0.004 where n = 106 and k = -0.2 where n = 39). These findings imply that the high level of agreement noted by the percentage agreement statistical test was largely a chance event. It is worth noting that the samples used in the current study were archived and could have degenerated. This could account for part of variations indicated by the Kappa test. In addition Kappa test is often affected in conditions where the prevalence rates are low (Hoelher, 2000). Thus, it is possible that the indication that the high level of agreement (74%) achieved by percentage agreement is inaccurate. However, fresh samples could be used to assess the interrater reliability of the three tests in future studies to obtain a batter estimate of the agreement due to chance.

There was a general agreement in the detection of the stem LFD LAMP products using gel electrophoresis and LFD. Notably, the positive and negative controls were positive and negative respectively in addition to positive results for samples previously found to be positive by sequencing indicating that the detection methods (gel electrophoresis and

LFD) could be used for the detection of amplification products considering their ease of use and cost of acquisition. On the contrary, SYBR® Green I dye could not differentiate some false positive products limiting its use as detection format in this assay. The percentage of false positives was 5% with not false positives. A possible explanation could be that intercalating dyes are specific and will bind to any double-stranded DNA including the primer-dimers (Mamiroli et al., 2007). In the current study, the false positives noted could be attributed to the formation of primer dimers, as explained by Cliften (2015). In the absence of caution, it is possible to erroneously interpret results. As such, the use of two or more detection formats to complement each other is desirable.

The restriction enzyme using the *Ndel* enzyme failed to digest the false positives confirming that the products were negative. Positive products gave the predicted amplicons of 137bp and 103bp respectively. Theoretically, LAMP test should not amplify non-specific products since amplification specificity is supposedly enhanced by using several primers. Nevertheless, when the test runs for too long or is inadequately optimized, spurious products are formed and account for false positive reactions. Determination of non-specific products is valuable since their presence reduces the amplification efficiency and ultimately the accuracy of the test. Higher levels of non-specific amplification have been associated with lower test sensitivities. Since most LAMP product detection formats are developed for visual inspection of results, a product confirmation step ought to be built into the test development protocol and/or a specific detection product format is recommended (Njiru, 2011). In this regard, the designed LFD format in this study showed superior specificity to the intercalating dyes. The dipstick format relies on a specific DNA sequence probe that binds to a specific complementary sequence in LAMP product. The lateral flow strips used in this study have duo detection ability for FITC and DIG-labeled products, but only the FITC was used. There is a non-specific faint line at DIG section that does not affect the results interpretation. Nonetheless, it indicates the need of using specific FITC-labeled strips only.

There is an agreement between the three tests in terms of detection sensitivity. All samples that were positive with nested PCR and SAM-1 LAMP test were also positive with stem LFD LAMP test indicating that the tests were detecting the same thing. Moreover, specificity of the stem LAMP test was confirmed through sequencing of product from four samples. Sequencing is a widely used laboratory technique for the confirmation of amplification products. As such, its application in this study served to demonstrate that the developed test had indeed detected the correct DNA. Notably, the stem LFD LAMP assay described here can further be improved by using a dipstick cartridge which allows insertion of the sample followed by a locking mechanism that cuts and pours the product directly into the LFD strip. The development of such technologies will eliminate the need to open the tube and potentially reduces contamination. Since stem LFD LAMP test is faster to perform and enjoys sensitivity levels higher or equal to PCR, the technique could form part of diagnostic algorithms for *Cryptosporidium* species detection where it can be used to select cases for further analysis.

Another aim of the current study was to develop a field applicable test for the detection of *Cryptosporidium*. In this regard, the benefits of the current test were compared to existing field diagnostic tests – microscopy. The current LFD Stem SAM-1 LAMP test not only allows batch processing but analyses 96 samples per test run compared to a much lower number of samples for microscopy. Lamp also requires less skilled personnel compared to microscopy, which requires personnel who can identify *Cryptosporidium* oocysts. The results of the current study also show that the LAMP test is more sensitive than microscopy and has lower levels of test-to-test variability. When compared to PCR, in terms of cost, the price of a PCR machine is significantly higher than that of a water bath. In addition, LAMP uses less power, given that it runs for a shorter time compared to PCR, and therefore, consumes less power. Cumulatively, these findings indicate that the LAMP based test is field applicable and achieves better outcomes compared to both PCR and Microscopy.

5.2 Conclusion

The current study was able to develop a more sensitive and specific LAMP based test compared to the published SAM-1 LAMP test. The Stem LFD SAM-1 LAMP test, in comparison to nested PCR and SAM-1 LAMP test, achieved higher analytical detection limits or analytical sensitivities. Moreover, stem LFD LAMP test achieved relatively shorter turn-around times. Pre-heating of the template was found to increase the sensitivity of this test by ten-fold. The use of multiple closely related DNA demonstrated that the test was highly specific. The omission of outer primers did not affect the sensitivity of the test.

Higher analytical sensitivity levels translated into high detection rates of positive clinical samples. Give that the stem LFD achieved higher detection rates and is faster to perform; the technique could form part of diagnostic tests for *Cryptosporidium* spp. detection where it can be used to select cases for further analysis. The results of gel-electrophoresis and dipstick were in agreement implying that they were detecting the same thing. However, SYBR green was unable to detect some false positives (due to primer dimers) making it difficult to use it solely as the detection method. Sequencing of positive products confirmed that LFD Stem SAM-1 LAMP Test had amplified *Cryptosporidium* spp.

5.2.1 Study limitations

The limited financial resources restricted the number of clinical samples that could be used in the study. Ideally, more clinical samples from different study centers ought to have been analyzed to better evaluate the test.

Very few samples were used in the comparison between gel electrophoresis and LFD. Considering the fact that the study was reporting the use of a dipstick for the detection of amplification products, more products ought to have been detected using the lateral flow dipstick.

5.3 Recommendations

i. Future studies ought to consider enrolling more centers to collect fresh samples for analysis, given that the use of archived samples could have affected the sensitivity of the test.

ii. Given that SYBR green suffers from the inability to detect false positives, the LFD test format can be advanced by the addition of a novel single-step reaction that will allow direct detection of product with the LFD strips without necessarily opening the tube needs to be considered. For instance, the use of a dipstick cartridge which allows insertion of the sample followed by a locking mechanism that cuts and pours the product directly into the LFD strip will contribute towards making Stem LFD LAMP a suitable complementary test to the current tests (microscopy and PCR) used in the detection of cryptosporidiosis, especially in resource-poor settings.

iii. Considering that the interrater reliability revealed that the agreement between the tests was to a large extent due to chance, it is important that more studies are conducted to would help increase the reliability of the test and the subsequent level of confidence.

iv. Future studies can be conducted without outer primers but with reaction accelerating loop and stem primers to increase the sensitivity and reduce the formation of primer dimmers that can be detected as false positives.

REFERENCES

Abdullahi, F., Naim, R., Taib, W., Saleh, A., Muazu, A., Aliyu, S., & Baig, A. (2015). Loop-mediated isothermal amplification (LAMP), an innovation in gene amplification: Bridging the gap in molecular diagnostics; A review. *Indian Journal of Science and Technology, 8(*17). DOI: 10.17485/ijst/2015/v8i17/55767.

Abubakar, I., Aliyu, S. H., Arumugam, C., Usman, N. K., & Hunter, P. R. (2007). Treatment of cryptosporidiosis in immunocompromised individuals: Systematic review and meta-analysis. *British Journal of Clinical Pharmacology, 63(*4), 387–393. http://doi.org/10.1111/j.1365-2125.2007.02873.x

Adamu, H., Petros, B., Zhang, G., Kassa, H., Amer, S., Yauyu, J., & Xiao, L. (2014). Distribution and clinical manifestations of *Cryptosporidium* species and subtypes in HIV/AIDS patients in Ethiopia. *PLOS Neglected Tropical Diseases, 8*(4), e2831, https://doi.org/10.1371/journal.pntd.0002831

Alves, M., Xiao, L., Sulaiman, I., Lal, A., Matos, O., & Antunes, F. (2003). Subgenotype Analysis of *Cryptosporidium* Isolates from humans, cattle, and zoo ruminants in Portugal. *Journal of Clinical Microbiology, 41(*6), 2744–2747. https://doi.org/10.1128/JCM.41.6.2744-2747.2003

Arunrut, N., Kampeera, J., Sirithammajak, S., Sanguanrut, P., Proespraiwong, P., Suebsing, R. & Kiatpathomchai, W. (2016). Sensitive visual detection of AHPND bacteria using Loop-Mediated Isothermal Amplification Combined with DNA-Functionalized gold nanoparticles as probes. *PLOS One, 11*(3), e0151769, https://doi.org/10.1371/journal.pone.0151769

Baker, D. (2008). *Flynn's Parasites of Laboratory Animals*. Hoboken, NJ: John Wiley & Sons.

Bakheit, M., Palomino, L., Thekisoe, M., Mbati, P., Ongerth, J., & Karanis, P. (2008). Sensitive and specific detection of *Cryptosporidium* species in PCR negative samples by loop-mediated isothermal DNA amplification and confirmation of

generated LAMP products by sequencing. *Veterinary Parasitology, 158*(1-2), 11-22.

Baveja, U., & Rewari, B. (2004). *Diagnosis and management of HIV/AIDS: A clinician's perspective*. New Delhi: BI Publications.

Baldursson, S., & Karanis, P. (2014). Waterborne transmission of protozoan parasites: Review of worldwide outbreaks- an update 2004-2010. *Water Research, 45*(20), 6603-6614.

Birx, Debbie. (2013). Analysis of the HIV epidemic in the highest burden countries. CGH-CHC. Retrieved from http://www.iapac.org/tasp_prep/presentations/TPSlon13_Closing_Panel_Birx.pdf

Bouzid, M., Hunter, P., Chalmers, R., & Tyler, K. (2013). *Cryptosporidium* pathogenicity and virulence. *Clinical Microbiology Reviews, 26*(1), 115-134.

Cacciò, S. M., &Widmer, G. (2014). *Cryptosporidium: parasite and disease*. Vienna: Springer.

Cao, L., Cheng, R., Yao, L., Yuan, S., & Yao, X. (2014). Establishment and application of a Loop-Mediated Isothermal Amplification method for simple, specific, sensitive and rapid detection of *Toxoplasma gondii*. *The Journal of Veterinary Medical Science, 76*(1), 9–14. https://doi.org/10.1292/jvms.13-0275\

Casemore, D., Armstrong, M., & Sanda, R. (1985). Laboratory diagnosis of cryptosporidiosis. *Journal of Clinical Pathology, 38*(12), 1337-1341.

Casemore, D. (1991). ACP broadsheet 128: June 1991 Laboratory methods for diagnosis of cryptosporidiosis. *Journ of Clinical Patholology, 44*(6), 445-451.

Caws, M., Tho, D., Mnh, P., Lan, N., Hoa, D., Torok, M., […] & Farrar, J. (2007). PCR-restriction length polymorphism for rapid, low-cost identification of isoniazid-resistant Mycobacterium tuberculosis. *Journal of Clinical Microbiology*, 45, 1789-1793.

CDC. (2015). *Cryptosporidium:* diagnosis and detection. Retrieved from http://www.cdc.gov/parasites/crypto/diagnosis.html

Chalmers, R., Campbell, B., Crouch, N., Charlett, A., & Davies, A. (2011). Comparison of diagnostic sensitivity and specificity of seven Cryptosporidium assays used in the UK. *Journal of Microbiology, 60,* 1598-1604.

Chalmers, R, (2014). *Cryptosporidium in Microbiology of waterborne diseases.* New York, NY: Academic Press.

Charan, J., & Biswas, T. (2013).How to calculate sample size for different study designs in medical research. *Indian Journal of Psychological Medicine, 35*(2), 121-126

Cheesbrough, M. (2005). *District laboratory practice in tropical countries*. Cambridge: Cambridge University Press.

Chen, M., Keithly, J., Paya, C., & Larusso, N. (2002). Cryptosporidiosis. *New England Journal of Medicine, 346*(22), 1723-1731.

Cheun, H., Chug, B., Won, M., Goo, B., Cho, S., Ji, M., & Lee, W. (2013). Development of a diagnostic kit to detect *Cryptosporidium parvum* and *Giardia lamblia*. *Osong Public Health and Research Perspectives*, *4*(3), 146-151.

Christen, R.S., Beser, J., Axen, C., & Lebbad, M. (2014). High applicability of a novel method for gp60-based subtyping of *Cryptosporidium meleagridis*. *Journal of Clinical Microbiology*, *52*(7), 2311-2319.

Cliften, P. (2015). Base calling, read mapping, and coverage analysis. London: Elsevier

Cobb, B. D., & Clarkson, J. M. (1994). A simple procedure for optimising the polymerase chain reaction (PCR) using modified Taguchi methods. *Nucleic Acids Research*, *22*(18), 3801–3805. http://doi.org/10.1093/nar/22.18.3801

Cochran W. G. (1963). *Sampling Techniques*. New York, NY: John Wiley and Sons, Inc.

Coupe, S., Sarfati, C., Hamane, S. & Derouin, F. (2005). Detection of *Cryptosporidium* and identification to the species level by nested PCR and restriction enzyme fragment length polymorphism. *Journal of Clinical Microbiology, 43*(3), 1017-1023.

Curtis, K., Rudolph, D. & Owen, S. (2008). Rapid detection of HIV-1 by reverse-transcription, loop-mediated isothermal amplification (RT-LAMP). *Journal of*

Virology Methods, 151, 264-270.

Desai, N., Sarkar, R. & Kang, G. (2012). Cryptosporidiosis: An under-recognized public health problem. *Tropical Parasitology, 2,* 91-8

Dhaliwal, B. & Juyal, P. (2013). *Parasitic zoonoses*. New Delhi. Springer.

Dhama, K., Karthik, K., Charkraborty, S., Tiwari, R., Kapoor, S., Kumar, A. & Thomas, P. (2014). Loop-mediated isothermal amplification of DNA (LAMP): A new diagnostic tool lights the world of diagnosis of aminal and human pathogens: A review. *Pakistan Journal of Biological Sciences, 17*(2); p. 151-166.

Dukes, J., King, D. & Alexandersen, S. (2006). Novel reverse transcription loop-mediated isothermal amplification for rapid detection of foot-and-mouth disease virus. *Archives of Virology,* 151, 1093-1106.

Fakruddin, M., Mannan, S., Abhijit, C., Reaz, M., Hossain, M., Islam, S., & Chowdhury, A. (2013). Nucleic acid amplification: Alternative methods of polymerase chain reaction. *Journal of Pharmacy and Bioallied Sciences, 5*(4); p.245-252.

Farrar, J., Hotez, P. J., Junghanss, T., Kang, G., Lalloo, D. G., White, N. J., & Manson, P. (2013). *Manson's tropical diseases*. London: Elsevier

Fayer, R. & Xiao, L. (2007). *Cryptosporidium and cryptosporidiosis*. Boca Raton, FL: CRC Press.

Gallas, L., Sotiriadou, J., Noack, M., Mahmoudi, M., & Karanis, P. (2016). *Giardia* and *Cryptosporidium* spp. dissemination during wastewater treatment and comparative detection via immunofluorescence assay (IFA), nested polymerase chain reaction (nested PCR) and loop-mediated isothermal amplification (LAMP). *Acta Tropica,* 158, 43-51.

Gandelman, O., Jackson, R., Kiddle, G., & Tisi, L. (2011). Loop-mediated amplification accelerated by stem primers. *Internal Journal of Molecular Sciences, 12,* 9108-24.

Gatei, W., Wamae, C., Mbae, C., Waruru, A., Mulinge, E., Waithera, I., […] & Hart, C. (2006).Cryptosporidiosis prevalence, genotype analysis and symptoms

associated with infections in children in Kenya. *The American Journal of Tropical Medicine and Hygiene, 75*(1), 78-82.

Karanis, P., Thekisoe, O., Kiouptsi, K., Ongerth, J., Igarashi, I., & Inoue, N. (2007). Development and preliminary evaluation of a loop-mediated isothermal amplification procedure for sensitive detection of *Cryptosporidium* oocysts in fecal and water samples. *Applied and Environmental Microbiology, 73*(17), 5660–5662. https://doi.org/10.1128/AEM.01152-07

Koonin, E. V., & Galperin, M. Y. (2003). *Sequence - evolution - function: Computational approaches in comparative genomics*. Boston, MA: Kluwer.

Hadfield, S., Robinson, G., Elwin, K. & Chalmers, R. (2011). Detection and differentiation of *Cryptosporidium* spp. in human clinical samples by use of Real-Time PCR. *Journal of Clinical Microbiology, 49*(3), 918–924. https://doi.org/10.1128/JCM.01733-10

Iqbal, A., Lim, Y., Surin, J. & Sim, B. (2012). High Diversity of *Cryptosporidium* Subgenotypes Identified in Malaysian HIV/AIDS Individuals Targeting gp60 Gene. *PLOS One, 7*(2), e31139. https://doi.org/10.1371/journal.pone.0031139.

Koczulla, K, & Gallotta, A. (2016). Lateral flow assays. *Essays in Biochemistry, 60*(1), 111-120.

Koloren, Z. (2017). LAMP detection of *Cryptosporidium* species in surface water samples collected from river Yesilirmak and stream Tersakan (Samsun-Amasya). *Anadolu University Journal of Science and Technology –C Life Sciences and Biotechnology, 6*(1). https://doi.org/10.18036/aubtdc.269434

Kumar, P., Gupta, V., Tiwari, A., & Kamle, M. (2016). *Current trends in plant disease diagnostics and management practices*. Switzerland: Springer

Kutima, H., Wasike, E., Muya, M. & Wamachi, A. (2015). Prevalence and risk factors associated with *Cryptosporidium* species infections in Bungoma county, Kenya. *Sky Journal of Medicine and Medical Sciences, 3*(3), 031-037

Lal, A., Michelle, C., Emily, F., Glass, K. & Kirk, M. (2015). Cryptosporidiosis: A disease of tropical and remote Areas in Australia. *PLOS Neglected Tropical Diseases,* http://dx.doi.org/10.1371/journal.pntd.0004078

LeChevallier, M., Giovanni. D., Clancy, J., Bukhari, Z., Rosen, J., Sobrino, J., & Frey, M. (2003). Comparison of method 1823 and cell culture-PCR for the detection of *Cryptosporidium* spp. in source waters. *Applied Environmental Microbiology, 69*(2), 971-9.

Lemon, S. M. (2007). *Global infectious disease surveillance and detection: Assessing the challenges--finding solutions: Workshop summary*. Washington, DC: National Academies Press.

Liao, C., Fu, J., Kao, C., Lee, Y., Chen, P., Chuang, T., […] & Fan, C. (2016). Prevalence of intestinal parasitic infections among school children in capital areas of the Democratic Republic of Sao Tome and Principle, West Africa. *African Health Sciences, 16*(3), 690-697.

Limor, J., Lal, A. & Xiao, L. (2002). Detection and differentiation of *Cryptosporidium* parasites that are pathogenic for humans by Real-Time PCR. *Journal of Clinical Microbiology*, *40*(7), 2335–2338. https://doi.org/10.1128/JCM.40.7.2335-2338.2002

Liu. D. (2014). *Manual of security sensitive microbes and toxins*. Boca Raton, Fl: CRC Press.

Liu, X., Xie, N., Li, W., Zhou, Z., Luhong, S., Cao, S. […] & Peng, G. (2015). Emergence of *Cryptosporidium hominis* monkey genotype II and novel subtype family Ik in the squirrel monkey (*Saimiri sciureus*) in China. *PLOS One, 10*(10); e0141450

Liu, X., Zhou, X., Zhong, Z., Chen, W., Deng, J., Niu, L., & Peng, G. (2014). New subtype of *Cryptosporidium cuniculus* isolated from rabbits by sequencing the Gp60 gene. *The Journal of Parasitology, 100*(4), 532–536. http://doi.org/10.1645/13-223.1McAdam, A. (2015). *Diagnostic Testing for*

Enteric Pathogens, An Issue of Clinics in Laboratory Medicine. London: Elsevier Health Sciences.

Mary, C., Chapey, C., Dutoit, E., Guyot, K., Jeddi, F., Paraud, C., […] & DErouin, F. (2013). Multicentric evaluation of a new Real-time PCR Assay or quantification of *Cryptosporidium* spp. and identification of *Cryptosporidium parvum* and *Cryptosporidium hominis*. *Journal of Clinical Microbiology, 51*(8), p.2556-2563.

Marrmiroli, N., & Maestri, E. (2007). *Polymerase chain reaction*. London: Elsevier

Mbae, Nokes, D., Mulinge, E., Nyambura, J., Waruru, A. and Kariuki, S. (2013). Intestinal parasitic infections in children presenting with diarrhea in outpatient and inpatient settings in an informal settlement of Nairobi, Kenya. *BMC Infectious Diseases, 13*(243).

McClatchey, K. (2001). *Clinical laboratory medicine*. Philadelphia: Lippincott Williams and Wilkins.

McHugh, M. (2012).Interrater reliability: The Kappa statistic. *Biochemia Medica, 22*(3), 276-282.

Meisel, J., Parera, D., Meligro, C., & Rubin, C. (1976). Overwhelming watery diarrhea associated with Cryptosporidium in an immunosuppressed patient. *Gastroenterology, 70*(6), 1156-1160.

Moghaddam, D., Azami, M., Salehi, R., & Salehi, M. (2008). The identification of *Cryptosporidium* spp. by PCR-RFLP analysis of the 18s rRNA gene. *International Journal of Infectious Diseases, 12*(1), e380-381.

Molloy, S., Smith, H., Kirwan, P., Nichols, R, Asaoulu, S., Connelly, L., & Holland, C. (2010). Identification of a high diversity of *Cryptosporidium* species genotypes and subtypes in a pediatric population in Nigeria. *The American Journal of Tropical Medicine and Hygiene, 82*(4), 608-613.

Monis, P., Giglio, S., & Saint, C. (2005). Comparison of SYTO9 and SYBR Green I for real-time polymerase chain and investigation of the effect of dye concentration on amplification and DNA melting curve analysis. *Analytical Biochemistry, 30*, 24-34.

Morgan, U., Pallant, L., Dwyer, W., Forbes, A., Rich, G., & Thompson, R. (1998). Comparison of PCR and microscopy for detection of *Cryptosporidium parvum* in human fecal specimens: Clinical Trial. *Journal of Clinical Microbiology, 36*(4), 995-998.

Mori, Y., Nagamine, K., Tomita, N., & Notomi, T. (2001). Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Biochemical and Biophysical Research Communications,* 289,150-154.

Muchiri, J., Ascollilo, L., Mutuma, M., Mutwiri, T., Honorine, D., Elena, N, Andrey, E., Cohen, S., Else, J. and Griffins, J. (2009).Seasonality of *Cryptosporidium* oocyst detection in surface waters of Meru, Kenya as determined by two isolation methods followed by PCR. *Journal of Water Health, 7*(1), 67-75.

Mwachari, C., Meier, A., Muyodi, J., Gatei, W., Waiyaki, P., & Cohen, C. (2003). Chronic diarrhea in HIV-1 infected adults in Nairobi, Kenya: Evaluation of risk factors and the WHO treatment algorithm. *AIDS, 17*(14), 2124-2126.

Nagamine, K., Hase, T., & Notomi, T. (2002). Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Molecular Cell Probes, 16,* 223-229.

Newman, R., Jaeger, K., Wuhib, T., Lima, A., Guerrant, R., & Sears, C. (1993). Evaluation of an antigen capture enzyme-linked immunosotbent assay for detection of *Cryptosporidium* oocysts. *Journal of Clinical Microbiology, 31*(8), 2080-2084.

Nichols, R., Campbell, B., & Smith, H. (2003). Identification of *Cryptosporidium* spp. oocysts in the United Kingdom noncarbonated natural mineral waters and drinking waters by suing a modified nested PCR restriction fragment length polymorphism assay. *Applied and Environmental Microbiology, 69*(7), 4183-4189.

Nichols, R., Connelly, L., Sullivan, C., & Smith, H. V. (2010). Identification of *Cryptosporidium* spp. and genotypes in Scottish raw and drinking waters during

a one-year monitoring period. *Applied and Environmental Microbiology, 76*(17), 5977–5986. http://doi.org/10.1128/AEM.00915-10.

Nichols, R., Campbell, B., & Smith, V. (2003). Identification of *Cryptosporidium* spp. oocysts in Unite Kingdom noncarbonated natural mineral waters and drinking waters by suing a modified nested PCR restriction fragment length polymorphism assay. *Applied and Environmental Microbiology, 69*(7), 4183-4189.

Njiru, Z., Mikosza, A., Matovu, E., Enyaru, J., Ouma, J., Kibona, S., […] & Ndungu, J. (2008). African trypanosomiasis: Sensitive and rapid detection of the sub-genus *Trypanozoon* by loop-mediated isothermal amplification (LAMP) of parasite DNA. *International Journal of Parasitology, 38*(5), 589-599.

Njiru Z. (2011). Rapid and sensitive detection of human African trypanosomiasis by loop-mediated isothermal amplification combined with a lateral-flow dipstick. *Diagnostic Microbiology and Infectious Disease, 69*(2), 205-9.

Njiru, Z (2012). Loop-mediated isothermal amplification technology: Towards point of care diagnostics. *PLOS Neglected Tropical Diseases, 6*(6): e1572, https://doi.org/10.1371/journal.pntd.0001572

Njiru, Z., Mbae, C., & Mburugu, G. (2017). Loop-mediated isothermal amplification test for *Trypanosoma gambiense* Group 1 with stem primers: A molecular xenomonitoring test for sleeping sickness. *Journal of Tropical Medicine,* Article ID 8630708, https://doi.org/10.1155/2017/8630708

Nolan, T., & Bustin, S. (2013). *PCR technology: Current innovations*. Boca Raton, FL: Florida: CRC Press.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. & Hase, T. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research, 28*(12), E63.

Notomi, T., Mori, Y., Tomita, N., & Kanda, H. (2015).Loop-mediated isothermal amplification (LAMP): principle, features, and future prospects. *Journal of Microbiology, 53*(1), 1-5.

Omuruyi, B., Nwodo, U., Udem, C., & Okwonkwo, F. (2014). Comparative diagnostic techniques for *Cryptosporidium* Infection. *Molecules*, 19(2), 2674-83.

Ortbald, K., Lozano, R., & Christopher, M. (2013). The burden of HIV: Insights from the global burden of disease study 2010. *AIDS*, *27*(13).

Ortega-Pierres, G. (2009). *Giardia and Cryptosporidium: From molecules to disease*. Wallingford, UK: CABI.

Ortega, Y. (2006). *Food-borne parasites*. New York, NY: Springer.

Parida, M., Sannarangaiah, S., Dash, P., Rao, P., & Morita, K. (2008). Loop-mediated isothermal amplification (LAMP): A new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. *Reviews in Medical Virology, 18,* 407-421.

Parida, M., Horioke, K., Ishida, H., Dash, P., & Saxena, P. (2005). Rapid detection and differentiation of Dengue virus serotypes by a real-time reverse transcription-loop-mediated isothermal amplification assay. *Journal of Clinical Microbiology, 43*, 2895-2903.

Puthawibool, T., Senapin, S., Klatpathomchai, W., & Fleget, T. (2009). Detection of shrimp infectious myonecrosis virus by reverse transcription loop-mediated isothermal amplification combined with a lateral flow dipstick. *Journal of Virolology Methods., 156*(1-2), 27-31.

Rafiei, A., Rashno, Z., Samarbafzadeh, A., & Shahram, K. (2014). Molecular characterization of *Cryptosporidium* spp. Isolated from immunocompromised patients and children. *Jundishapur Journal of Microbiology, 7*(4), e9183.

Ruecker, N., Hoffman, R., Chalmers, R., & Neumann, N. (2011). Detection and resolution of Cryptosporidium species and species mixtures by genus specific nested PCR restriction fragment length polymorphism analysis, direct sequencing, and cloning. *Applied and Environmental Microbiology, 77*(12), 3998-4007.

Qian, X., & Lloyd, R. (2003). Recent developments in signal amplification methods for in situ hybridization. *Diagnostic Molecular Pathology, 12*(1), 1-13.

Rekha, K., Puttalakshmamma, C., & Placid, D. (2016). Comparison of different diagnostic techniques for the detection of cryptosporidiosis in bovines. *Veterinary World, 9*(2), 211-215.

Rossle, N. F., & Latif, B. (2013). Cryptosporidiosis as threatening health problem: A review. *Asian Pacific Journal of Tropical Biomedicine, 3*(11), 916–924. http://doi.org/10.1016/S2221-1691(13), 60179-3.

Robertson, L. (2014). *Cryptosporidium as a foodborne pathogen*. New York, NY: Springer.

Sahoo, R., Sethy, K., Mohapatra, S., & Panda, D. (2016). Loop-mediated isothermal amplification: An innovative gene amplification technique for animal diseases. *Veterinary World, 9*(5), 465-469.

Sajid, M., Kawde, N., & Daud, M. (2015). Designs, formats, and applications of lateral flow assay: A literature review. *Journal of Saudi Chemical Society, 19*(6), p.689-700.

Sharma, R., Arya, S., Deepak, S., Atin, S., Pradeep, K., Kumar, N., & Pathania, R. (2014). Identification of novel regulatory small RNAs in *Acinebacter baumanni*. *PLOS One, 9*(4), e93833, https://doi.org/10.1371/journal.pone.0093833

Singh, S. (2014). *Emerging and re-emerging human infections: Genome to infectome*. Hoboken, NJ: Wiley-Blackwell.

Siobhan, M., & Tzipori, S. (2008). Cryptosporidiosis in Children in Sub-Saharan Africa: A lingering challenge. *Clinical Infectious Diseases, 47*(7), 915-921.

Slack, J. M. W. (2012). *Essential Developmental Biology*. Somerset: Wiley.

Snelling, J., Xiao, L., Ortega-Pierres, G., Lowery, J., Moore, E., […] & Dooley, J. (2007). Cryptosporidiosis in developing countries. *Journal of Infection in Developing Countries, 1*(3), 242-256.

Squire, S., & Ryan, U. (2017).*Cryptosporidium* and *Giardia* in Africa: Current and future challenges. *Parasites and Vectors, 10*(195), doi: 10.1186/s13071-017-2111-y

Stensvold, C., Elwin, K., Winiecka-Krusnell, J., Chalmers, R., Xiao, L. & Lebbad, M. (2015). Development and application of a gp60-based typing assay for *Cryptosporidium viatorum*. *Journal of Clinical Microbiology, 53*(6), 1891–1897. https://doi.org/10.1128/JCM.00313-15

Stubaum, D., Reed, C., Homer, P., Jost, H., Marshall, M., & Sterling, C. (2001). Species-specific, nested PCR restriction fragment length polymorphism detection of single *Cryptosporidium parvum* oocysts. *Applied Environmental Microbiology, 67*(6), 2665-2668.

Su, Y., Yang, Y., Peng, Q., Zhou, D., Chen, Y., Wang, Z., […] & Que, Y. (2016).Development and application of a rapid and visual loop-mediated isothermal amplification for the detection of *Sporisorium scitamineum* in sugarcane. *Scientific Reports, 3,* 23994, DOI: 10.1038/srep23994

Sulaiman, I., Morgan, U., Thompson, R., Lal, A., & Xiao, L. (2000). Phylogenetic relationships of *Cryptosporidium* parasites based on the 70-kilodalton Heat Shock Protein (HSP70) gene. *Applied and Environmental Microbiology, 66*(6), 2385–2391. https://doi.org/10.1128/AEM.66.6.2385-2391.2000

Sunit, S. (2014). *Emerging and re-emerging human infections: Genome to infectome.* Hoboken, NJ: Wiley-Blackwell.

Tellevik, M., Sabrina, J., Blomberg, B., Hjollo, T., Maselle, S., Langeland, N., & Hanevik, K. (2015). Prevalence of *Cryptosporidium parvum, hominis, Entamoeba histolytica* and *Giardia lamblia* among young children with and without diarrhea In Dar es Salaam, Tanzania. *PLOS Neglected Tropical Diseases, 9*(10), e0004125, http://dx.doi.org/10.1371/journal.pntd.0004125.

Tomita, N., Mori, Y., &Notomi, T. (2008). Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nature Protocols, 3*(5), 877-882.

Trietsch, D., and Baker, K. (2013). *Principles of sequencing and scheduling*. Hoboken, N.J: Wiley.

Tumwine, J., Kekitilnwa, A., Bakeera, K., Ndeezi, G., Downing, R., Feng, K., […] & Tzipori, S. (2005). Cryptosporidiosis and microsporidiosis in Ugandan children with persistent diarrhea with and without concurrent infection with the Human Immunodeficiency Virus. *American Journal of Tropical Medicine and Hygiene, 73*(5), 921-925.

Tzipori, S., Angus, K., Campbell, I., & Gray, E. (1980). *Cryptosporidium*: evidence for a single-species genus. *Infection and Immunity, 30*, 884-886.

Tzipori, S, & Ward, H. (2002). Cryptosporidiosis: Biology, pathogenesis and disease. *Microbes and Infection, 4*, 1047-1058.

Ungar, B. (1990). Enzyme-linked immunosorbent assay for detection of *Cryptosporidium* antigens in fecal specimens. *Journal of Clinical Microbiology,* 28(1), 2491-2495.

Vohra, P., Sharma, M. & Chaudhary, U. (2012). A comprehensive review of diagnostic techniques for the detection of *Cryptosporidium parvum* in stool samples. *Journal of Pharmacy*, *2*(5), 15-26.

Wang, L., Zhang, H., Zhao, X., Zhang, L., Zhang, G., Guo, M., […] & Xiao, L. (2013). Zoonotic *Cryptosporidium* species and *Enterocytozoon bieneusi* genotypes in HIV-positive patients on Antiretroviral therapy. *Journal of Clinical Microbiology*, *51*(2), 557-563.

Wanyiri, J., Kanyi, H., Maina, S., Wang, D., Steen, A., Ngugi, P., […] & Ward, H. (2014). Cryptosporidiosis in HIV/AIDS patients in Kenya. Clinical features, epidemiology, molecular characterization, and antibody responses. *American Journal of Tropical Medicine and Hygiene*, *91*(2): 319–328. doi: 10.4269/ajtmh.13-0254

Weber, R., Bryan, T., Bishop, H., Wahlquist, S., Sullivan, J., & Juranek, D. (1991).Threshold of detection of *Cryptosporidium* oocysts in human stool specimens: Evidence for low sensitivity of current diagnostic methods. *Journal of Clinical Microbiology, 29*(7), 1323-1327.

Weitzel, T., Dittrich, S., Mohi, I., Adusu, T., & Jelinek, T. (2006). Evaluation of seven commercial antigen tests for *Giardia* and *Cryptosporidium* in stool samples. *Clinical Microbiology Infections, 12*(7), 656-659.

Wiser, M. (2012). *Protozoa and human diseases.* New York, NY: Garland Science.

Widerstrom, M., Schonning, C., Lillia, M., Lebbad, M., Liung, T., Allestam, G., […] & Lindh, W. (2014). Large outbreak of *Cryptosporidium hominis* infection transmitted through the public water supply, Sweden. *Emerging Infectious Diseases, 20*(4), 581-580.

Xiao, L., Escalante, L., Yang, C., Sulaiman, I., Escalante, A., Montali, R., […] & Lai, A. (1999). Phylogenetic analysis of *Cryptosporidium* parasites based on the small subunit rRNA gene locus. *Applied Environmental Microbiology, 65*(4), 1578-1583.

Xiao, L., Fayer, R., Ryan, U., & Upton, S. (2004). *Cryptosporidium* taxonomy: Recent advances and implications for public health. *Clinical Microbiology Reviews, 17*(1), 72-97.

Yang, C., & Heinsohn, P. (2007). *Sampling and analysis of indoor microorganisms.* New York, NY: John Wiley and Sons.

Yoder, J., & Beach, M. (2010). *Cryptosporidium* surveillance and risk factors in the United States. *Experimental Parasitology, 124*(1), 31-39.

APPENDICES

KENYA MEDICAL RESEARCH INSTITUTE

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KEMRI/RES/7/3/1 **August 11, 2015**

TO: **MAMBA TIMOTHY,**
PRINCIPAL INVESTIGATOR

THROUGH: **THE DIRECTOR, CMR,**
NAIROBI

Forwarded 13/8/15

Dear Sir,

RE: **SSC PROTOCOL NO. 2891 (*RESUBMISSION 2 OF INITIAL SUBMISSION*): DEVELOPMENT AND EVALUATION OF A LATERAL FLOW LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) TEST FOR THE DETECTION OF CRYPTOSPORIDIUM OOCYSTS.**

Reference is made to your undated letter. The KEMRI/Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised study documents on August 5, 2015.

This is to inform you that the Committee notes that the issues raised at the 232nd meeting of the KEMRI Ethics Review Committee held on 21st October, 2015 have been adequately addressed.

Consequently, the study is granted approval for implementation effective this **11th August 2015** for a period of one year. Please note that authorization to conduct this study will automatically expire on **August 10, 2016.** If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **June 29, 2016**.

You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of SERU and you should advise SERU when the study is completed or discontinued.

You may embark on the study.

Yours faithfully,

FOR

PROF. ELIZABETH BUKUSI,
ACTING HEAD,
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT

In Search of Better Health

Appendix 1: KEMRI Scientific Ethics Review Unit (SERU) approval of the research study

Appendix 1: KEMRI SERU

KENYA MEDICAL RESEARCH INSTITUTE

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KEMRI/SSC/103070 8th September, 2014

Timothy Mamba

Thro'
Director, CMR
NAIROBI

forwarded

REF: SSC No. 2891 (Revised) – Development and Evaluation of a Lateral Flow Loop-Mediated Isothermal Amplification (LAMP) Test for the Detection of *Cryptosporidium* Oocysts

I am pleased to inform you that the above mentioned proposal, in which you are the PI, was discussed by the KEMRI Scientific Steering Committee (SSC), during its **218th** meeting held on **2nd September, 2014** has since been approved for implementation by the SSC.

Kindly submit 4 copies of the revised proposal to SSC within 2 weeks from the date of this letter i.e., 22nd September, 2014 for onward transmission to the ERC.

We advise that work on this project can only start when ERC approval is received.

FOR: Sammy Njenga, PhD
SECRETARY, SSC

Appendix 2: KEMRI Scientific Steering Committee (SSC) approval for the implementation of the proposed research study

Appendix 2: KEMRI SSC

Appendix 3: Peer reviewed research article