A STUDY ON CRYPTOSPORIDIOSIS AMONG HIV-POSITIVE PATIENTS PRESENTING WITH DIARRHOEA FROM RURAL AND URBAN AREAS IN RWANDA

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DECLARATION

This project is my original work and has not been presented in any institution leading to the award of a degree or any other award.

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DEDICATION

To my beloved wife and children

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I am deeply indebted to Almighty God who has guided me through the whole period of my studies.

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ABBREVIATION

AIDS	Acquired Immunodeficiency Syndrome
ART	Antiretroviral treatment
ARV	Antiretroviral
Bp	Base pair
C	Cryptosporidium
CD4	Cluster of differentiation
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
H_2SO_4	Sulfuric Acid
HIV	Human Immunodeficiency Virus
IFN-γ	Interferon gamma
KUTH	Kigali University Teaching Hospital
MDCK	Madine-Darby Canine Kidney
NaCl	Sodium chloride
NTZ	Nitazoxamide
OI	Opportunistic infection
PCR	Polymerase Chain Reaction
pH	Le potentiel hydrogen
RDH	Ruhengeri District Hospital
RFLP	Restricted Length Polymorphism
RMH	Rwanda Military Hospital
RT-PCR	Real-time polymerase chain reaction
Spp	Species
USA	United State of America
ZN	ZiehlNeelsen staining

ABSTRACT

Introduction: Cryptosporidiosis is a disease caused by *Cryptosporidium species* and it causes prolonged cholera-like diarrhoea in HIV infected patients once their CD4 cells count falls below 200cells/µl. This study sought to determine the prevalence of *Cryptosporidium spp.* infection in HIV positive patients with diarrhoea using a PCR method as gold standard and to compare the sensitivity and specificity of PCR to those of direct stool smears and stool smears after formal ether concentration when both are stained by modified ZN method. The study was conducted at three hospitals in Rwanda and involved 377 HIV positive patients presenting with diarrhoea from outpatients and inpatients.

Results: The prevalence of *Cryptosporidium spp.* infection in HIV positive patients was 34.2% (129/377) after using the PCR method. Direct stool smear stained by modified ZN staining showed sensitivity of 66.6% and specificity of 100% while stool smears from formal ether concentration showed sensitivity of 98.4% and specificity of 100 %. *Cryptosporidium* infections were detected more frequently in urban 38.2% (100/262) than rural areas 25.2% (29/115), there was a significant association between *Cryptosporidium spp.* and in those who reported having had diarrhoea for more than 30 days 72% (18/25) and in patients with CD4 cell count of less than 200 cells per µl 51.8% (128/247). *Cryptosporidium infection* was not associated with occupation, marital status, sex, age, education, water source and to eating places ($p>0.05$) in this study.

Conclusion: This study showed high association between HIV positive patients presenting with diarrhoea and *Cryptosporidium spp.* and recommend modified ZN method for staining of stool smears made after formal ether concentration when testing for *Cryptosporidium spp.*

DEFINITIONS

Diarrhoea: Excessive and frequent evacuation of water faeces, usually indicating gastrointestinal distress or disorder [1].

Cryptosporidium species: A genus of protozoans of the order Coccidia that are parasitic in the gut of many vertebrates including humans and that sometimes cause diarrhoea especially in individuals who are immunocompromised (as in AIDS) [2].

Cryptosporidiosis (*Cryptosporidium* infection): Is an illness caused by tiny C*ryptosporidium* parasites. When *Cryptosporidium* parasites enter the body, they travel to the small intestine and then burrow into the walls of the intestines. Later, cryptosporidia are shed in the faeces [3]. In most healthy people, a *Cryptosporidium* infection produces about of watery diarrhoea and the infection usually goes away within a week or two. If one has a compromised immune system, a *Cryptosporidium* infection can become life threatening without proper treatment [3].

Oocyst: Infective stage of *Cryptosporidium* parasites to the human and other vertebrates. It is also a resistant stage of the life cycle of coccidial parasites. It contains a zygote and under appropriate conditions sporulates to become a mature infective oocyst [4]. It may also remain infective for long periods in dry conditions [4].

CHAPTER 1: INTRODUCTION

Opportunistic parasitic infections are among the most serious infections in human immunodeficiency virus (HIV) positive patients and claim number of lives every year [5]. Chronic diarrhoea in HIV-positive patients with CD4+ T-cell counts <200/µl has high probability of association with intestinal parasitic infections [5]. When the CD4 cell count falls below 200 cells/µl, there is an irreversible breakdown of immune defence mechanism, and the patient becomes prey to a variety of human opportunistic pathogens like bacteria, viruses, fungi, and parasites [6]. Identification of these parasitic infections may play an important role in administration of appropriate therapy and reduction of mortality and morbidity in these patients [5].

Cryptosporidium is a coccidian protozoan parasite that infects humans and animals [7]. The *Cryptosporidium*, a small, obligate intracellular parasite, has emerged as an important cause of chronic life-threatening diarrhoea [8]; causing prolonged and cholera-like diarrhoea in HIV infected patients [7]. However, in immune-competent persons, it may cause a short-term diarrheal illness that resolves spontaneously. The infection occurs through the ingestion of environmentally resistant oocysts, (faecal-oral route) [9-11].

It causes short term gastrointestinal illnesses in immune competent patients with debilitating diarrhoea, which are often accompanied by severe abdominal cramps, weight loss, anorexia, malaise and low grade fever which lasts for a longer duration in immune deficient patients [11].

Lately, the strong association between cases of cryptosporidiosis and immune-deficient individuals (such as those with AIDS) has brought *Cryptosporidium spp.* to the forefront as ubiquitous human pathogen[12]. Reported Cryptosporidiosis prevalence is 3-4% in the USA [13], 3.5-22.4% in Brazil and about 50-60% in Africa and Haiti [14]. However, this parasite has been identified in up to 46% of HIV infected patients world-wide in other studies [15]. In Rwanda, there is no published data on the prevalence of cryptosporidiosis in HIV positive patients with diarrhoea, and the only study reported on cryptosporidiosis in Rwanda was in 1987 on clinical and epidemiological features of the disease [16].

The diagnosis of cryptosporidiosis is based on several methods but the cheapest and simplest one is fresh smear preparation of stool with modified ZN staining technique. However, this staining technique has been found to have a low sensitivity detecting only 46-67% of the persons with *cryptosporidium* oocysts [17]. The stool sedimentation using formal ether concentration technique before then smear preparation, and staining with modified ZN techniques has been shown to increase the sensitivity and specificity for detection of *Cryptosporidium* oocysts up to 95% [5, 18, 19]. The sensitivity and specificity of modified ZN staining results using the smears prepared from formal ether concentration technique was measured by the comparison of the PCR results, which was taken as the gold standard. The literatures have showed that PCR technique has sensitivity and specificity of 100% [20]. The early detection of *Cryptosporidium spp.* infection is very important in preventing complications and in prolonging a healthy life in HIV-positive patients [9, 10].

1.1 Research Questions

What is the prevalence of *Cryptosporidium spp.* in HIV positive patients with diarrhoea?

What is the sensitivity and specificity of modified ZN in detection of C*ryptosporidium spp*. using the sediment from formal ether concentration method?

1.2 Study Objectives

1.2.1 General Objective

To determine the prevalence of *Cryptosporidium spp.* in HIV positive patients with diarrhoea in an urban and a rural region in Rwanda, and to evaluate the sensitivity and specificity of modified ZN method using the deposit from formal ether concentration technique during detection of *Cryptosporidium spp.* using PCR technique as the gold standard.

1.2.2 Specific Objectives

1. To determine the prevalence of *Cryptosporidium spp*. in HIV positive patients with diarrhoea in an urban and a rural area in Rwanda

2. To access sociodemographic and socioeconomic factors associated with *Cryptosporidium spp.* infection
3. To compare the results of direct smear and smear of sediment obtained using a formal ether concentration when both are stained by modified ZN method
4. To determine the sensitivity and specificity of modified ZN methods in the diagnosis of *Cryptosporidium spp*. in Rwanda

1.3 Study Justification

Since the discovery of acquired immunodeficiency syndrome (AIDS), many studies have demonstrated that intestinal parasites are frequently associated with severe diarrhoea in HIV infected patients. HIV infection, a worldwide phenomenon, is a serious problem in the present day. *Cryptosporidium species* are the coccidian protozoan parasites endemic to many regions including the Caribbean, Central and South America, Africa and Southeast Asia. They are common in tropical and subtropical environments where Rwanda is situated. The infections are common in immunosuppressed patients, particularly those with AIDS living in tropical areas and these opportunistic infections are accelerating disease progression in HIV-positive individuals leading to quick death [9, 11].

Currently there is no study that has been published to show the magnitude of cryptosporidium in HIV positive patients in Rwanda hence the need for this which will go a long way in helping health authorities to make informed decisions. This will help to implement the modified ZN using the smear from formal ether concentration technique as the best and appropriate diagnostic method at all levels of health delivery system. It will also help to know the prevalence of *Cryptosporidium spp.* infection in HIV positive patients with diarrhoea.

Studies have shown that the early detection of the causative parasites plays a significant role in implementing timely and correct treatment, which relieves the patients' symptoms and also prevents recurrences [21].

CHAPTER 2: LITERATURE REVIEW

2.1 Epidemiology

Diarrhoea is a major cause of morbidity and mortality in HIV infected individuals. In the coming years there is likely to be an increase in the number of HIV/AIDS deaths, with worrying projections of 6.5 million deaths in 2030 and HIV/AIDS being the main burden of disease in some developing countries by 2015 [22]. Opportunistic enteric parasitic infections are encountered in 30-60% of HIV sero-positive patients in developed countries and in 90% of patients in developing countries [22]. Once the $CD4^+$ cell count drops below 200cells/µl, patients are considered to have developed AIDS, with the risk of an AIDS-defining illness or opportunistic infection significantly increasing. One of this opportunistic enteric parasites encountered in HIV/AIDS patients is *Cryptosporidium species* [22]. Cryptosporidiosis, a zoonotic disease which is caused by *Cryptosporidium species*, is a recognized coccidial protozoan infection causing severe protracted watery diarrhoea in humans [23]. Many species of *Cryptosporidium* can infect humans but the most common species responsible for watery diarrhoea is *C. parvum* and *C. hominis*.

2.2 Transmission

Cryptosporidial infection is transmitted from faecally contaminated food and water, from animal to person contact, and via person to person contact [24]. This occurs when one accidentally ingests oocysts, the infective stages.

Food and water

There have been six major outbreaks of cryptosporidiosis in the United States as a result of contamination of drinking water [4]. One major outbreak in Milwaukee in 1993 affected over 400,000 persons, currently there is no published literature on outbreaks on Cryptosporidiosis in Africa. Outbreaks such as these usually result from drinking water taken from surface water sources such as lakes and rivers [4]. Swimming pools and water park wave pools have also been associated with outbreaks of cryptosporidiosis. Also, untreated groundwater or well water public drinking water supplies can be sources of contamination [4].

Food can also be a source of transmission, when either an infected person or an asymptomatic carrier contaminates a food supply. The first documentation of this type of infection occurred at a county fair in Maine, where children who drank apple cider contaminated by animal faeces developed cryptosporidiosis [4]. The oocysts do not survive cooking, but food contamination can occur in beverages, salads, or other foods not heated or cooked after handling [4].

Animal to person transmission

Transmission of *C. parvum* from household pets is extremely rare, but there is a definite correlation between calves and humans. Studies conducted in the United States of America have shown approximately 50% of calves shed oocysts and the pathogen is present on upwards of 90% of all dairy farms [4].

Person to person transmission

Cryptosporidium transmission occurs at a high frequency in day-care centres, where infants or younger children are clustered within classrooms, share toilets and common play areas, or necessitate frequent diaper-changing [25]. Day-care employees can become easily infected by *C. parvum* through careless diaper-changing or through washing the laundry of infected children. Day-care workers can then spread the pathogen to their families at home [25]. Nosocomial settings are also a major forum for cryptosporidial transmission. There have been several reports of both transmission from patients to health care staff and patient-to-patient transmission [26].

2.3 Biology of *Cryptosporidium Species*

Life cycle

The *C. parvum* life cycle includes both asexual multiplication and sexual reproduction [2, 27, 28]. Oocyts are ingested by the host, after which sporozoites excyst. Excystation usually requires reducing conditions, pancreatic enzymes or bile salts, although spontaneous excystation in aqueous conditions can also occur [2, 27]. Sporozoites are infective, and attach to epithelial cells, after which they become enveloped by the host apical cell membrane, reside in a resulting parasitophorous vacuole that contains

membrane components from both the host and *C. parvum*, and differentiate into the spherical trophozoites. *C. parvum* resides intracellularly but outside of the cytoplasm, which contrasts with other intracellular pathogens (e.g. toxoplasma) that reside in parasitophorous vacuoles within the cytoplasm *C. parvum* is thought to obtain nutrients from the host through a feeder organelle that is visible as membrane folds, some of which are connected with small vesicles of host cytoplasm [2, 27, 28].

During maturation of the *C. parvum* trophozoite, asexual multiplication occurs and results in the formation of a type I schizont that contains six to eight merozoites. Rupture of the schizont results in the release of merozoites that, in turn, can invade adjacent host epithelial cells, where they develop subsequently into type I or type II schizonts [28]. Type I schizonts contain merozoites important in asexual multiplication, whereas type II schizonts initiate sexual reproduction (gametogony) by differentiating into male microgametes or female macrogametes. Male microgametes release microgametes that can fertilize the macrogametes inside the female macrogamete. The oocysts that are generated then sporulate. There are two types of oocysts, thin-walled oocyts and thick-walled oocyts. It is thought that thin-walled oocysts excyst and reinfect the host, whereas thick-walled oocysts exit the intestinal tract and can subsequently infect a new host [2, 27, 28].

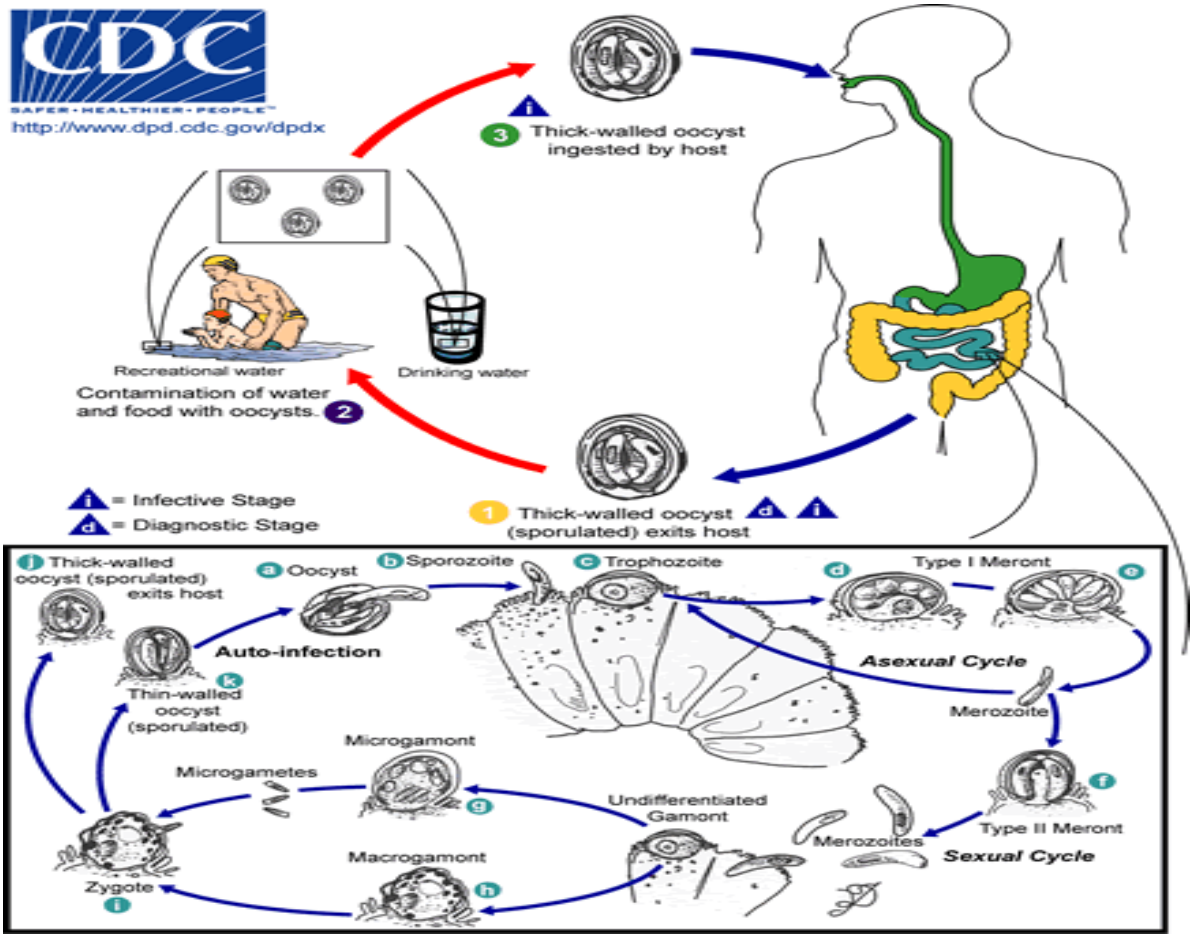

Figure 1: Life cycle of *Cryptosporidium species* (CDC Photo; DPDx)

2.4 Clinical Presentation

Clinical manifestations in infected humans follow an incubation period, which generally ranges from two to 14 days [2]. They include watery and often profuse diarrhoea, as well as abdominal cramps, nausea, vomiting, weight loss, low-grade fever weakness, malaise, fatigue, cholecystitis especially in HIV infected patient, hepatitis, pancreatitis, reactive arthritis, respiratory symptoms, disseminated disease [2, 29]. In immunocompetent individuals, disease is usually self-limited in duration, whereas the duration of illness is prolonged in the immunocompromised host [2]. In addition, in severe infection, malabsorption can be present due to decreased absorptive surface and this can contribute to the wasting syndrome in infected AIDS patients. Immunocompromised patients who

develop bile duct infection can present with jaundice secondary to biliary tract obstruction or with symptoms of pancreatitis [2].

2.5 Pathogenesis

The pathogenic mechanisms by which *Cryptosporidium spp.* causes diarrhoea, malabsorption and wasting are poorly understood [30]. The initial host–parasite interactions of attachment, invasion and parasitophorous vacuole formation are complex processes that involve multiple parasite ligands and host receptors. These interactions have been best studied in apicomplexans such as *Toxoplasma*, *Plasmodium* and *Eimeria* [30]. Invasive "zoite" stages of apicomplexans possess specialized secretory organelles (rhoptries, micronemes and dense granules) collectively known as the apical complex. Increasing recognition of *Cryptosporidium* as an emerging human pathogen has led to the identification of surface and/or apical complex proteins (such as CSL, GP900, p23/27, TRAP C1, GP15, CP15, CP60/15, cp47, gp40/45 and gp15/Cp17) that have features in common with those of other apicomplexans and that are implicated in mediating these interactions [30]. *C. parvum* infection, glucose-stimulated sodium absorption was inhibited and this paralleled the extent of villous and epithelial cell, damage increased mucosal prostaglandin production (e.g., PGE2 and PGI2), which can inhibit neutral NaCl absorption and result in secretory diarrhoea [2]. The mechanisms that lead to increased prostaglandin production and the cellular source of the prostaglandins are not known [2]. In addition, resident and recruited leukocytes in the mucosa (e.g. macrophages) have the potential to produce high levels of prostaglandins, and may also be a source of prostaglandin production during *C. parvum* infection [2]. Alterations in intestinal permeability may also play a role in the diarrhoea in *C. parvum*-infected individuals [2]. In this regard, increased levels of gamma interferon (IFN-γ) produced during infection could increase intestinal permeability and decrease epithelial barrier function [2].

2.6 Laboratory Diagnosis

Laboratory diagnosis of cryptosporidiosis relies on several methods including special staining techniques stool smears (Modified ZN staining), the use of ELISA for the detection of *Cryptosporidium* antigen in stools, Antibody detection for *Cryptosporidium*,

Cultural methods, Histopathological examination of jejunal biopsy specimens and Molecular methods. During this study two methods will be performed (modified ZN staining and the PCR method). Modified ZN staining is preliminary test and PCR method is gold standard to confirm the results tested by modified ZN method.

Modified ZN staining

Faecal smears made directly from the stool sample or from the concentration deposit are stained by modified Ziehl-Neelsen staining, then decolorize by 2.5% sulfuric acid oracid alcohol (1% HCl in methanol), counterstain with 0.4% malachite green or 1% methylene. The stained smears are screened under high power (40X) and oil immersion (100X) objectives of light microscope for identification of the coccidian parasites - *Cryptosporidium species* [17, 21, 22].

Detection of antigen directly from faecal specimens

The use of ELISA for the detection of *Cryptosporidium* antigen in stool (Ridascreen®-Biopharm) has sensitivity of 66-100% and specificity of 93-100% [22, 31].

Antibody detection

Antibody detection for *cryptosporidium* is useful in population-based studies to determine the prevalence of infection within a community. It cannot diagnose acute infection. Commercial kits available are ProSpecT ELISA, IDEIA, Colour VUE, having a sensitivity of 95-97% and specificity of 98-100% [22, 32].

Culture methods

Monkey and rabbit kidney cell lines (Vero and RK-13), human foetal lung fibroblast cell lines (MRC-5) and Madine–Darby canine kidney canine kidney cell lines (MDCK) are employed for microsporidial culture. [22, 33, 34].

Molecular methods

PCR is a biochemical technology in molecular biology used to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence [22]. Although it is more expensive and time-consuming, PCR has been shown to have a more superior sensitivity for the detection of *Cryptosporidium species* as compared to conventional staining and microscopy [22].

PCR diagnostic test for detection of *C. parvum* DNA has been widely used with primers CPBDIAGF and CPBDIAGR. This detects <10 oocyst in crude samples. Molecular base pair standard is a 100-bp ladder [22, 35].

A TaqMan PCR assay targeting the 18S ribosomal DNA (rDNA) has allowed sensitive detection of *Cryptosporidium species* in stools. Nested PCR and Real Time RT-PCR are employed to assess oocyst viability. PCR-restricted fragment length polymorphism (RFLP) analysis can also be done directly from stool samples and the prevalent genotypes of a particular region can be identified [36, 37].

Histopathological examination

Histopathological examination of jejunal biopsy specimens from infected individuals with *Cryptosporidium species* shows mild to moderate acute inflammation of the lamina propria and surface epithelial disarray [33].Staining of tissue sections by the Warthin-Starry staining method is an effective diagnostic tool for the microscopic detection of microsporidia and has better diagnostic capabilities than the haematoxylin and eosin stain. Plastic embedded tissue sections by Toluidine blue stain is also a suitable alternative [22, 33].

2.7 Treatment

No safe and effective therapy for cryptosporidial enteritis has been successfully developed. Since cryptosporidiosis is a self-limiting illness in immunocompetent individuals, general, supportive care is the only treatment for the illness [38]. Oral or intravenous rehydration and replacement of electrolytes may be necessary for particularly

voluminous, watery diarrhoea. Oral rehydration treatment can include Gatorade, bouillon, or oral rehydration solution, containing glucose, sodium bicarbonate, and potassium [38, 39].

For immunocompromised patients with cryptosporidiosis, the aminoglycoside paromomycin continues to be one of the few antimicrobial agents that remain consistently in clinical use [12, 40]. One of the newer chemotherapeutic agents to be evaluated is nitazoxanide (NTZ). NTZ is a nitrothiazolyl-salicylamide derivative with broad-spectrum parasiticidal activity against protozoa, nematodes, trematodes and cestodes [41, 42]. Its reported efficacy against these parasites led to trials of the drug for cryptosporidiosis [43]. Patients with AIDS-associated cryptosporidiosis in Mali reported a 95% reduction in oocyst excretion [42]. AIDS patients in Mexico reported parasitological cure (no oocysts detected in fecal samples) [44]. Of these, 86% of the patients also reported resolution of diarrhoea [44]. In immunocompetent individuals with diarrhoea due to cryptosporidiosis in Egypt [45], using 3 days of treatment with nitazoxanide, 80% showed clinical improvement in symptoms after 7 days and 67% had no oocysts detected in their stool. Clearly, further clinical trials of this drug alone and in combination with other drugs, such as paromomycin or azithromycin, in immunocompetent as well as immunocompromised patients are warranted [27, 45].

2.8 Prevention and Control

Prevention of exposure in at high risk individuals, proper disposal of the faeces to prevent contamination of the soil and water, boiling/filtering drinking water along with improved personal hygiene might go a long way in preventing the enteric parasitic infections [22, 29].

CHAPTER 3: METHODOLOGY

3.1 Study Area

This study was conducted at Rwanda Military Hospital (RMH), Kigali University Teaching Hospital (KUTH) and at Ruhengeri District Hospital (RDH). The RMH and KUTH are main tertiary referral hospitals located in Kigali, the capital city of Rwanda, whereas RDH is one of the main district hospitals located in Northern Province of the country. In the three hospitals, adult inpatients or outpatients with HIV/AIDS are mainly followed up within Internal Medicine departments by medical and nursing teams as well as social workers and psychologists. The RMH and KUTH mainly receive patients living in the capital and surrounding areas or those who referred from other parts of the country. RDH receives patients living in Northern Province, mainly within its catchment area covering a population of approximately three hundred thousand [46]. In the three hospitals, the laboratory staff are regularly retrained and supervised by staff deployed from the National Reference Laboratory in Kigali.

The modified ZN technique is the method used to diagnose *Cryptosporidium species* in all the laboratory departments of the hospitals that were involved in this study. When and if further confirmatory tests such as the use of PCR is required samples are sent to National Reference Laboratory which is the only facility capable of doing such tests, due to lack of equipment in the referring laboratories.

3.2 Study Design

This was a hospital-based prospective cross-sectional study. It was conducted between August 2014 and June 2015.

3.3 Study Population

The study participants were HIV positive patients presenting with diarrhoea at the three hospitals. Both outpatients and inpatients from these facilities participated in the study.

3.4 Inclusion Criteria

Patients had to be 21 years old and above, who indicated their consent to participate in the study by signing a written informed consent form.

3.5 Exclusion Criteria

All HIV negative patients, HIV positive patients without diarrhoea and HIV positive patients with diarrhoea below 21 years were excluded from participating in the study.

3.6 Data Collection Procedure

Permission to conduct the study was obtained from the hospital authorities of all the three hospitals. With the assistance of a nurse from the departments of internal medicine and ARV service, the study objectives and requirements were explained to all potential participants in Kinyarwanda, after which they were requested to participate in the study. Those who indicated their willingness to participate were requested to indicate this by providing their written informed consent. A standard questionnaire was then used to obtain demographic and clinical information from the patients' file consisting of geographical area where they patients live, their level of education, occupational status, marital status, age, sex, socioeconomic status, duration of diarrhoea, and their CD4 cell counts. Stool samples were then collected in the clean dry sterile plastic containers for laboratory examination.

3.7 Methods and Materials

3.7.1. Specimens collection and handling

Faecal samples collected from inpatients and outpatients at three hospitals in Rwanda and involved three hundred seventy-seven HIV positive patients presenting with diarrhoea. Each specimen was labelled with information on the patients' number, age and sex and

then was immediately transported to the laboratories of respective hospitals. Each sample was divided into 2 portions. The first portion was preserved in aliquots in ethanol at 4ºC until processing by PCR [47]. The second portion was used to prepare direct stool smears and stool smears after formal ether concentration technique before staining each smear using modified ZN staining technique.CD4 cell counts for the patients with results tested within the last six months were also filled in the questionnaire. Blood samples were collected for CD4 cell count test from patientswhoseCD4 cell count results were older more than six months.

3.7.2. Formal-ether concentration technique

This formal-ether concentration technique consists in fixing parasites' eggs, larvae, oocysts, and spores without changing their morphology so that they are no longer infectious. In this technique, faecal debris is extracted into the ethyl acetate phase of the solution while parasitic elements are sedimented at the bottom [20].

A portion of each fresh stool sample was taken and processed. Briefly, 1 g of stool was placed in a clear 15 mL conical centrifuge tube containing 7 mL formalin saline by using applicator stick, stool was mixed with it. The resulting suspension was filtered through a sieve into another conical tube. After adding 3ml of diethyl ether to the formalin solution, the content was centrifuged at 3200 rpm form 3 minutes. The supernatant was poured away and the tube was replaced in its track. Finally smears were prepared from the sediment then fixed with methanol and stained by modified ZN staining [5].

3.7.3. ZN staining technique

Cryptosporidium are usually not detected during direct examination of specimens and feacal smear made directly from the stool sample [20], but laboratory diagnosis of cryptosporidiosis traditionally relies on special staining techniques, such as modified ZN stain [22]. By using the modified ZN technique to stain smear from the concentration deposit increases the chance of observation the infective stage of oocysts, which are usually 4–6 μm in size [20].

Faecal smear from the concentration deposit are stained by modified ZN staining, then decolorize by 2.5% sulfuric acid or acid alcohol (1% HCl in methanol), counterstain with 0.4% malachite green or 1% methylene. The stained smears are screened under high power (40X) and oil immersion (100X) objectives of light microscope for identification of the coccidian parasites - *Cryptosporidium species* [17, 18, 21, 22].

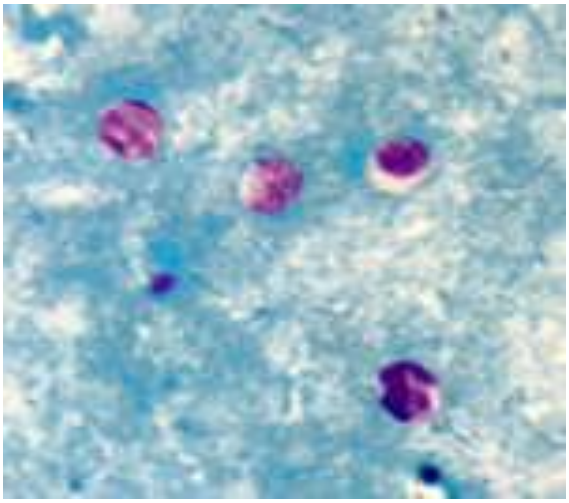

Figure 2: *Cryptosporidium* oocysts in a modified acid-fast stain. (CDC Photo; DPDx).

3.7.4. Polymerase Chain Reaction (PCR) technique

PCR techniques have the advantages of improving sensitivity and specificity because it amplifies a specific region of a DNA (the DNA target). Compared to the modified ZN stain, PCR was found to be the most efficient [20]

DNA Extraction

Cryptosporidium spp. DNA was extracted from *Cryptosporidium oocysts* in stool samples by first pipetting 200µl of the liquid stool sample into a 2ml micro-centrifuge tubes. The tubes were then placed on ice. QIAGEN QIAamp DNA stool mini kit was used for extraction of the parasite DNA according to the manufacturer's manual instructions. Approximately 1.4ml of Buffer ASL was added to each stool sample and vortexed continuously until the sample was thoroughly homogenized. The suspension

was then heated at 70°C for 5 minutes before vortexing for 15 seconds. The samples were then centrifuged at full speed for 1 minute so as to pellet the parasite.

Approximately 1.2ml of the supernatant was pipetted into a new 2ml micro-centrifuge and the tube containing the pellet discarded. OneinhibitEX tablet was added to each sample and vortexed immediately and continuously until the tablet was completely suspended. The suspension was then incubated for 1 minute at room temperature to allow the inhibitors to adsorb to the inhibitEX matrix. The samples were then centrifuged at full speed for 3 minutes in order to pellet inhibitors bound to the inhibitEX matrix. The resulting supernatant was pipetted into a new 1.5ml micro-centrifuge tube and the pellet discarded. The sample was again centrifuged at full speed for 3 minutes.

Lysis was performed immediately after by pipetting 15ul of proteinase K into a new 1.5ml micro-centrifuge tube. Two hundred (200) µl of the supernatant from the above step was then pipetted into the 1.5 ml tube containing the proteinase K before adding 200ul buffer AL and 2µl of the internal control from the PCR kit. The mixture was then vortexed for 15 seconds followed by incubation at 70°C for 10minutes. Two hundred (200) µl of absolute ethanol was added to the lysate and mixed by vortexing. Spin columns supplied with the extraction kit were placed in a 2ml collection tube before transferring all the lysate from the above steps to the spin column. The columns in the collection tubes were then centrifuged at full speed for 1 minute and the resulting filtrate discarded. Five hundred (500) ml of Buffer AW1 was then added to the columns and centrifuged before discarding the filtrate again. Five hundred (500) µl of buffer AW2 was also added to the columns, centrifuged and the filtrate discarded as above. The spin columns were then placed in a new 2ml collection tube and centrifuged at full speed for 1 minute in order to eliminate any ethanol residuals in the DNA. Finally, elution of the DNA was performed by placing the spin column in a new 1.5ml centrifuge tubes and adding 200µl of buffer AE. The tubes were then incubated at room temperature for 1 minute before collecting the DNA by centrifuging at full speed for 1 minute.

The extracted DNA was stored at -20°C awaiting PCR.

Amplification and detection of the *Cryptosporidium spp.* by real time PCR

Amplification and detection of the *Cryptosporidium spp.* DNA was done using multiplex real-time PCR kit as per the manufacturer's instruction (Fast-Track Diagnostics). The kit is intended for use as an aid to the evaluation of infections with *Entamoeba histolytica, Cryptosporidium spp. (including C. parvum, C. hominisand C. meleagridis) and Giardia lamblia (G. la lamblia, G. intestinalis,* and *G. duodenalis).* This was accomplished by adding 1.5ul of the primer and probe mix to 12.5µl of the PCR buffer and 1ul of the Taq polymerase to create a master mix of 15µl per sample. 10µl of the extracted DNA was then added to create a total reaction volume of 25µl.The tubes were closed and vortexed briefly before loading onto the real time thermo cycler (Rotor Gene 5plex-QIAGEN). Amplification was then performed by denaturing the DNA at 95°C for 10 minutes followed by 40 cycles comprising of cycle denaturation of DNA at 94°C for 8 seconds and annealing of the primers and probes to the DNA combined with elongation at 60°C for 34 seconds. Fluorescence reading was performed at the end of each cycle at wavelengths of 670nm (red channel) for detection of the *Giardia lamblia*, 550nm (yellow channel) for detection of *Entamoeba histolytica* 610nm (Orange channel) Cryptosporidium spp. and 520nm (green) for detection of the internal control.

A positive and negative control was included in each run as a quality assurance check.

3.7.5. CD4 cell count test

The flow cytometer (BD facs count) was used to measure CD4 cell counts for patients who did not have the results of them.

3.8 Interpretation of Results

The preliminary reports were the presence of the *Cryptosporidium* oocysts after modified ZN staining technique which were observed under oil-immersion objectives (×100) as small pink to red spherules on pale green background and registered on data collection sheets. The PCR test showed that the sample qualified for reporting only if the positive controls included in the run were all positive and the negative controls were all negative. Also all the samples must have had an amplification in the internal control channel which

was an indication of a successful DNA extraction process. Secondly, a sample was reported as being positive for either of the parasite if it showed an exponential fluorescence trace in the respective wavelength or channel.

3.9 Quality Assurance

Known positive, negative controls and internal control, which come in the PCR kits were run concurrently with test samples. This process assured reliability of the results.

3.10 Data Management

Data was stored and maintained in Epi Info 3.5.1 (CDC) then transferred and analysed using SPSS statistical software version 20 (IBM® SPSS® Statistics 20). Data collected in hard copies were kept in lockable cabinets where only the researcher has access to maintain confidentially. Information stored in Epi Info databases was password protected from unauthorized individual. All records were identified by study ID numbers to maintain confidentiality. All this information was kept at Rwanda Military Hospital archive department where only authorized persons had access to this information.

3.11 Data Analysis and Presentation

Sensitivity and specificity values were calculated using the following formula [48]

$$Sensitivity = \frac{TP}{TP+FN};\ Specificity = \frac{TN}{TN+FP};\ Positive\ predictive\ value = \frac{TP}{TP+FP};\ Negative\ predictive\ value = \frac{TN}{TN+FN}$$

TP: True positive results; TN: True negative results; FN: False negative results; FP: False positive results. These results were defined based on modified ZN microscopy results versus PCR (Gold standard test).

Statistical analysis of the prevalence was calculated as total number of infected samples/total number of samples examined ×100. Fisher's exact and chi-square tests were used to determine the relationship between the presence of *Cryptosporidium spp.*in the patients who provided the stool samples and other parameters such as geographical area,

occupational status, marital status, age, sex, socioeconomic status, diarrhoea duration and CD4 cell counts. The P value <0.05 was considered statistical significant.

3.12 Sample Size

The sample size was calculated using [DEFF*Np(1-p)]/[($d^2/Z^2_{1-\alpha/2}$*(N-1)+p*(1-p)] formula[49] derived from Epi version 2.3.1[49]. An estimated human cryptosporidiosis prevalence of 32.4% was used based on a Ugandan study [50].

Sample size (**n)=[DEFF*Np(1-p)]/[($d^2/Z^2_{1-\alpha/2}$*(N-1)+p*(1-p)]= 337**

Sample size **(n)= 337**

Population size (for finite population correction factor or fpc)(N):1000000

Hypothesized % frequency of outcome factor in the population (p): 32.4%+/-5

Confidence limits as % of 100 (absolute +/-%)(d):5%

Design effect (for cluster surveys-DEFF):1

3.13 Ethical Considerations

Approval to conduct this study was obtained from Rwanda National Ethical Review Committee and Kenyatta National Hospital/University of Nairobi Ethic and Research committee (Appendix 9, 10). Written informed consent (Appendix 1) was obtained from each patient prior to enrolment into the study. Personal identification information obtained from the recruited patients was kept strictly confidential. No identifiers were used on the data collection tools. Instead only unique numbers were used. Data were accessed only by authorized persons who had a password for the computer and/or a key for the storage cupboard. Detailed report regarding the outcome results of the study will be sent to the National HIV program and Ministry of Health for their prompt action.

CHAPTER 4: RESULTS

4.1 Demographic information

A total of 377 HIV positive patients presenting with diarrhoea were enrolled in this study. Demographic characteristics of this study population are shown in Table 1. The majority of the study participants 225 (60%) were male. The largest proportion of the study participants 160(42%) were married, and 167(44%) were aged 40-49 years. Among the total number of HIV positive patients presenting with diarrhoea 115(31%) were from the rural area. Most of the participants 240(64%) had primary level education. Those with secondary school education or above were 115(30%). Only 22(6%) had no formal education. Half of the study participants 189(50%) were labourers (farmers, skilled or unskilled manual workers), with 141(38%) of the participants being sellers and clerks (street vendors and service sellers).

Table 1: Socioeconomic and demographic characteristics of participants in three hospitals in Rwanda, August 2014 to March 2015

Characteristics	n=377	%
Residence		
Rural	115	31
Urban	262	69
Patients classification		
Outpatients	235	62
Inpatients	142	38
Formal education		
Secondary or above	115	30
Primary	240	64
No education	22	6
Occupation*		
Professional	9	2
Seller and clerk	141	38
Labourers	189	50
Unemployed	38	10
Marital status		
Married	160	42
Not married but living with partner	80	21
Single/separated/divorced/widow	134	36
Missing values	3	1
Age in years		
20-29	28	7
30-39	105	28
40-49	167	44
≥50	77	21
Sex		
Female	151	40
Male	225	60
Missing values	1	0

*****Professional (people trained in their area of operation i.e. Have university and college level); seller and clerk (street vendor and selling services), labourers (farmer, skilled and unskilled manual worker).

4.2 *Cryptosporidium* infection in HIV positive patients presenting with diarrhoea

One hundred and twenty-nine (129) out of the 377 patients examined were found to be infected with *Cryptosporidium spp.* giving an overall prevalence of *cryptosporidium spp.* infection in the study population of 34.2% and was obtained using PCR method. One hundred (38.2%) of the patients from the urban areas who presented with diarrhoea were found to be infected with *Cryptosporidium spp.* compared to 29(25.2%) of the patients from the rural areas. The difference in the prevalence of *Cryptosporidium spp*. infection between patients coming from the urban areas and those from the rural areas was statistically significant ($\chi 2$ test; p=0.01). See Table 2.

Table 2: *Cryptosporidium spp.* infection in relation to residence among patients with diarrhoea

Variables	Diagnosed with *Cryptosporidium spp.* n=377				p-value
	Yes	%	No	%	
Residence					
Rural	29	25.2	86	74.8	0.01
Urban	100	38.2	162	61.8	

Age-specific prevalence of *Cryptosporidium spp.* infection is shown in Table 3. The age group with the highest prevalence of infection was 40-49 years, while those with the lowest prevalence of infection were aged 50 years and above. However, the difference in the prevalence of infection in relation to age was not statistical significant ($\chi 2$ test; p=0.77). See table 3.

Table 3: *Cryptosporidium spp.* infection in relation to age among patients with diarrhoea

Variables	Diagnosed with *Cryptosporidium spp.* n=377				p-value
	Yes	%	No	%	
Age in years					
20-29	9	32.1	19	67.9	0.77
30-39	36	34.3	69	65.7	
40-49	61	36.5	106	63.5	
≥ 50	23	29.9	54	70.1	

Overall, females had a slightly higher prevalence of *Cryptosporidium* infection 53(35.1%) than males 76(33.8%). However, this difference was not statistically significant ($\chi 2$ test; p=0.79). See Table 4.

Table 4: *Cryptosporidium spp.* infection in relation to gender among patients with diarrhoea

Variables	Diagnosed with *Cryptosporidium spp.* n=377				p-value
	Yes	%	No	%	
Sex					
Female	53	35.1	98	64.9	0.79
Male	76	33.8	149	66.2	

The prevalence of *Cryptosporidium spp.* infection among the study population did not differ significantly with marital status ($\chi 2$ test; p=0.79). See Table 5.

Table 5: *Cryptosporidium spp.* infection in relation to marital status among patients with diarrhoea

Variables	Diagnosed with *Cryptosporidium spp.* n=377				p-value
	Yes	%	No	%	
Marital status					
Married	56	35	104	65	0.79
Not married but living with partner	29	36.2	51	63.8	
Single/separated/divorced/widow	43	32.1	91	67.9	

Cryptosporidium spp. infection was found to be more prevalent among patients who reported using treated water 125 (35.1%) than among those who reported using untreated water 4 (19%). Similarly, this difference was not statistically significant ($\chi 2$ test; p=0.13). See Table 6.

Table 6: *Cryptosporidium spp.* infection in relation to water source among patients with diarrhoea

Variables	Diagnosed with *Cryptosporidium spp.* n=377				p-value
	Yes	%	No	%	
Source of water supply†					
Treated water	125	35.1	231	64.9	0.13
Untreated water	4	19	17	81	

†Treated water: piped water supplied by Rwanda's Water Sanitation Corporation, untreated water: water from spring, surface water (river, lakes, dam or rainy water) or tube well or bore hole.

The prevalence of *Cryptosporidium spp.* infection was lowest 84 (31.1%) among patients who reported that they rarely ate restaurant food. However, the prevalence of infection in relation to the patients' frequency of eating in restaurants was not statistically significant ($\chi 2$ test; p=0.13). See Table 7.

Table 7: *Cryptosporidium spp.* infection in relation to eating places among patients with diarrhoea

Variables	Diagnosed with *Cryptosporidium spp.* n=377				p-value
	Yes	%	No	%	
Using restaurant food‡					
Often	30	42.3	41	57.7	0.13
Sometimes	15	41.7	21	58.3	
Rarely	84	31.1	186	68.9	

‡Often (eating in the restaurant > 2 times a week); sometimes (eating in the restaurant 1- 2 times a week or a month); rarely (eating in the restaurant less than once a month).

The prevalence of *Cryptosporidium spp.* infection was highest among patients who indicated that they were professionals 7(58.3%) with the lowest prevalence among those who reported that they were unemployed 38(26.3%). However, there was no statistically significant differences in the prevalence of *Cryptosporidium spp.* infection in relation to the occupation of the HIV positive patients presenting with diarrhoea ($\chi 2$ test; p=0.16). See Table 8.

Table 8: *Cryptosporidium spp.* infection in relation to occupation among patients with diarrhoea

Variables	Diagnosed with C*ryptosporidium spp.* n=377				p-value
	Yes	%	No	%	
Occupation*					
Professional	7	58.3	5	41.7	0.16
Seller and clerk	57	37	97	63	
Labourers	55	31.8	118	68.2	
Unemployed	10	26.3	28	73.7	

*Professional (people trained in their area of operation i.e. Have university and college level); seller and clerk (street vendor and selling services), labourers (farmer, skilled and unskilled manual worker).

The prevalence of *Cryptosporidium spp.* infection was highest 18(72%) among patients who reported having had diarrhoea for more than 30 days, followed by those who reported having had diarrhoea for between 15 and 30 days 35(68.6%) , and lowest

76(25.2%) among those who reported having had diarrhoea for l to 14 days. (χ2 test; p=0.01). See Table 9.

Table 9: *Cryptosporidium spp.* infection in relation to duration of diarrhoea

Variables	Diagnosed with *Cryptosporidium spp.* n=377				p-value
	Yes	%	No	%	
Diarrhoea duration					
1-14 days	76	25.2	225	74.8	0.01
15-30 days	35	68.6	16	31.4	
>30 days	18	72	7	28	

1-14 days (Acute diarrhoea), 15-30 days (persistent diarrhoea) and >30 days (chronic diarrhoea).

The prevalence of *Cryptosporidium spp.* infection was highest 128(51.8%) among the patients with CD4 cell count of less than 200 cells per µl, followed by those with CD4 cell count 200-350 cells per µl. None of the patients with CD4 cells above 350 cells per µl was found to be infected with *Cryptosporidium spp.* infection. The difference in prevalence of *Cryptosporidium spp.* infection in relation to CD4 cell count was statistically significant (χ2 test; p=0.01). See Table 10.

Table 10: *Cryptosporidium spp.* infection in relation to CD4 cell count among patients with diarrhoea

Variables	Diagnosed with *Cryptosporidium spp.* n=377				p-value
	Yes	%	No	%	
CD4 cells per µl					
>350	0	0	28	100	0.01
200-350	1	1	101	99	
<200	128	51.8	119	48.2	

The prevalence of *Cryptosporidium spp.* infection was higher among inpatients 113(79.6%) than among the outpatients 16(6.8%). This difference was statistically significant (χ2 test; p=0.01). See Table 11.

Table 11: *Cryptosporidium spp.* infection in relation to patient's location in the hospitals

Variables	Diagnosed with *Cryptosporidium spp.* n=377				p-value
	Yes	%	No	%	
Patients location in the Hospitals					
Outpatients	16	6.8	219	93.2	0.01
Inpatients	113	79.6	22	20.4	

4.3 Comparison of PCR and modified ZN test for diagnosis of *Cryptosporidium spp*

Using PCR as the gold standard, direct stool smears examination after modified ZN staining found 86/377 specimens to be true positives. There were no false positives. Direct stool smears examination also showed 291/ 377 smears to be negative. Of these, 248/291 were true negatives and 43/291 were false negatives. Using stool smears after formal ether concentration method and staining with modified ZN stain, there were 127/377 true positives with no false positives. This method also showed 250/377 to be negative out of which 248/250 were true negatives and 2/250 were false negatives. Thus, direct stool smears examination after staining with modified ZN stain had a sensitivity of 66.6% and specificity of 100%, while stool smears examination after formal ether concentration method and staining using modified ZN had a sensitivity of 98.4% and specificity of 100%. Direct stool smears stained by modified ZN had a negative predictive value (NPV) of 85% and a positive predictive value (PPV) of 100% whereas stool smears after formal ether concentration method and also staining using modified ZN had a NPV of 99.2% and PPV of 100%. See table 12.

Table 12: A comparison of direct stool smears and stool smears from formal ether concentration both stained by modified ZN method with PCR technique

Type of test		**Diagnosis of Cryptosporidium spp.**		**Total**	**Sensitivity**	**Specificity**	**PPV**	**NPV**
		Positive	Negative					
Smear from fresh stool*	Positive	86	0	86	66.6%	100%	100%	85%
	Negative	43	248	291				
Total		129	248	377				
Smear after formal ether concentration†	Positive	127	0	127	98.4%	100%	100%	99.2%
	Negative	2	248	250				
Total		129	248	377				

* Direct stool smears stained by modified Ziehl-Neelsen staining

† Stool smears after formal ether concentration stained by modified Ziehl- Neelsen staining

CHAPTER 5: DISCUSSION

The *Cryptosporidium spp.* is one of the major opportunistic infections (OIs) in HIV positive patients especially who have CD4 below 200cells/µl [5, 6]. A previous study of cryptosporidiosis in Rwanda found a prevalence of 2.6% among patients with and without diarrhoea, and with and without HIV infection [51]. The present study focused on *Cryptosporidium spp.* among HIV positive patients in Rwanda aged 21 years and above, and presenting with diarrhoea. The high *Cryptosporidium spp.* prevalence of 34.2% found in this population is similar to what has previously been observed in other African countries. A study conducted by Salyer *et al* in Uganda found a prevalence of infection of 32.4% [50]. Similarly a study by Pedersen *et al* in Tanzania found a prevalence of infection of 44-60% [52]. Another study conducted by Alemu *et al* in Ethiopia found a prevalence of infection of 43% [5]. Also closely related study conducted by Acquah *et al* in Ghana found a prevalence of infection of 33.3% [53]. In this study prevalence of Cryptosporidiosis in HIV positive patients also falls within the range of 8-48% observed among African AIDS patients with diarrhoea in a study conducted by Masarat *et al* [14]. The high prevalence of *Cryptosporidium ss*p. infection in these developing countries may be attributed to the fact that HIV infection is often diagnosed late in this part of the world leading to many of the patients having low CD4 cell counts as a result of not being on antiretroviral treatment.

The present study found a higher prevalence of *Cryptosporidium spp.* infection in patients from urban as compared to rural settings. These findings are in agreement with the findings of a study conducted in Kenya [54] where the prevalence of *Cryptosporidium spp.* in HIV positive patients were 2.9 higher in urban residents when compared to peri-urban residents and 2.2 times higher when compared to rural residents. This could be attributed to the fact that a higher number of participants were coming from urban area and could easy access to health facility.

There was no statistical association observed between age and *Cryptosporidium spp.* infection just as was the case with gender and marital status groups. A study conducted by Salyer *et al* in Uganda found no association between the prevalence of

Cryptosporidium spp. infection and patients' age [50]. Likewise a study conducted by Certad *et al* in Venezuela showed that there was no association between *Cryptosporidium spp.* infection, gender and age [55]. Furthermore, Aminu *et al* in Nigeria found that *Cryptosporidium spp.* infection was not associated with neither marital status, sex and age [56].

The observed lack of association between Cryptosporidium *spp.* infection and water source in HIV positive patients presenting with diarrhoea has previously been described by Nyamwange *et al* in Kenya [54]. However, in contrast, Aminu *et al* in a study conducted in Nigeria found a significant association between water source and *Cryptosporidium spp.* infection [57]. In that study, the patients whose source of drinking water was the tap had a significantly lower prevalence of infection (58.9%) compared to those whose source of drinking water was rivers (100%), and patients who boiled water before drinking had lower prevalence of infection than those who did not. The reason for the latter is plausible since boiling of water kills oocysts of *Cryptosporidium spp.* parasites.

The present study found no association between *Cryptosporidium spp.* infection with eating in restaurants. This finding supports the finding in a study conducted by Ajjampur *et al* in India which found significant risk factors for *Cryptosporidium spp.* infection being keeping of pigs, dogs and storage of cooked food for later consumption [57]. No other study has previously shown an association between eating in restaurants and risk of acquiring *Cryptosporidium spp.* infection.

The present study showed no significant association between the prevalence of *Cryptosporidium spp.* infection and occupation. Aminu *et al* showed no significant association between *Cryptosporidium spp.* infection and occupation, a surrogate for socioeconomic factors. Similar findings have been also observed in a study conducted by Ibrahim *et al* in Nigeria which showed there was no significant association between *Cryptosporidium spp.* infection and occupation [58].

The higher prevalence of *Cryptosporidium spp.* infection observed among patients with chronic diarrhoea (>30 days) is not surprising since cryptosporidiosis is known to be associated with prolonged diarrhoea especially in the immunocompromised individuals. A study conducted in Ethiopia by Girma *et al* found a strong association between the prevalence of *Cryptosporidium spp.* infection and duration of diarrhoea. In that study, of the 34.3% of the HIV positive patients with *Cryptosporidium spp.* infection 40% had chronic diarrhoea, 32.1% had acute diarrhoea and only 4% had no diarrhoea.

The strong association found between *Cryptosporidium spp.* infection and low CD4 cell counts in the present study has previously been described. In the study by Nyamwaya *et al* conducted in Kenya the mean CD4 counts among patients with cryptosporidiosis was 3 times lower than in those without cryptosporidiosis, showing a significant association between cryptosporidiosis and the CD4 counts (OR = 0.9918, 95% CI = 0.9874 -0.9961, p = 0.0002)[54]. Similarly, the study conducted by Aminu *et al* in Nigeria found a significant association (p= 0.000) between *Cryptosporidium spp.* infection and CD4cells count. In that study, patients with CD4cells count between101-200 had the highest (75.9%) prevalence of infection while patients with CD4cells count between 901-1000 had the lowest (0.6%) prevalence of infection [56].

The observed higher prevalence of *Cryptosporidium spp.* infection among the inpatients than among the out-patients can be attributed to fact that a larger proportion (51.8%) of the inpatients had CD4 <200cells/µl compared to 1% of the out-patients.

The present study found a higher sensitivity of microscopic examination of ZN stained stool smears prepared after formal ether concentration (98.4%) compared to directly prepared stool smears (66.6%). A higher sensitivity of microscopic examination of ZN stained stool smears prepared after formal ether concentration method has previously been described. A study by Tahvildar-Biderouni *et al* found that the stool smears made after formal ether concentration method stained with modified ZN staining had 94% sensitivity and 100% specificity [17]. Another study conducted in South Africa by Omoruyi *et al* found less sensitivity of 46.2% and specificity of 88.9% when direct smears microscopy was used to detect *Cryptosporidium spp.* infection in stool [20]. The present study showed that microscopic examination of stained stool samples after

processing it using formal ether concentration method was more sensitive than direct stool examination after staining using the same method. This suggests that formal ether concentration method should be the preferred method of processing stool samples to be examined for *Cryptosporidium spp.* infection especially in resource-constrained countries, which cannot afford the more sensitive diagnostic tests such as direct fluorescent antibody technique or molecular diagnostic techniques such as DNA probes and PCR tests, after evaluation between PCR as gold standard in this study and modified ZN stain methods for the detection of *Cryptosporidium* spp. infection, PCR was found to be most efficient as compared to the other diagnostic techniques utilized because PCR techniques have the advantages of improved sensitivity and specificity by amplifying a specific region of a DNA (the DNA target). However, PCR methods have limited applicability at point-of-care or low-resource settings due to their costs, infrastructure needs, and the high technical expertise involved [20].

This study has few limitations. Initially we aimed at increasing the number of study sites in order to increase the sample size and therefore the power of sample but this was not feasible due to financial constraints. Increasing study sites would have also provided further insight into the epidemiological profile of *Cryptosporidium* spp. Infection among HIV positive patients at health facility level. Thus, our findings cannot be generalized to the whole country since the study was limited to only three hospitals. Similarly, this study included only adult population, which does not reflect the real situations in all HIV-infected patients. Other studies including paediatric patients should be considered in the future to.

CHAPTER 6: CONCLUSIONS & RECOMMENDATIONS

6.1. Conclusions

In this study there was high prevalence rate 33.9% of *Cryptosporidium spp*. infection among HIV positive patients presenting with diarrhoea and low CD4 counts of cells less than200cells/µl compared to patients with CD4 counts of cells above 200cells/µl.

The study also showed high sensitivity (98.2%) for *Cryptosporidium spp*. infection in smears made from formal ether concentration method as compared to smears made from direct stool preparation (66.6%) and both stained by modified ZN method. However, the two methods showed similar high specificity of 100%.

6.2. Recommendations

There is need to routinely use of stool smears made from formal ether concentration method and stain with modified ZN method for diagnosis of suspected cases of *Cryptosporidium spp*. infection as this method is affordable and easy to perform.

There is also a need for a large scale comparison study to establish the leading cause of opportunistic infections (OIs) among HIV positive patients with and without diarrhoea as during this study there was other parasites like *Entamoeba hystolytica* and *Giardia lamblia* found.

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APPENDICES

Appendix 1

Consent form for research participants

Title of the study: Study on cryptosporidiosis among HIV-positive patients presenting with diarrhoea from a rural and an urban areas in Rwanda.

Investigator

MURANGWA Anthere **Phone:** +250788406065/+254711166242

Purpose

You are invited to participate in this research study. The purpose of this study is to determine the prevalence of cryptosporidium spp. in HIV positive patients with diarrhoea, to determine the sensitivity and specificity of modified ZN method using PCR method as golden standard in Rwanda.

Subjects

You are invited to participate in this study if you are only HIV positive patient with diarrhoea.

Procedures

If you consent to participate, you will be requested to provide 4gr of stool specimens to be used for diagnosis of cryptosporidium species, these quantities of stool specimens will be measured by precision balance and 4ml of blood for CD4 counts.

Risks

No any risk has been identified.

Benefits

You will get modified ZN and PCR test results free of charge and data from this study may help health facility, planner and other researchers to offer the best services in diagnosis and management of HIV positive patients with diarrhoea.

Confidentiality

Your stool and blood samples will be assigned a code number and the key to the code will be maintained by the principal investigator. Records of this study will be stored in a locked file cabinet at Rwanda Military Hospital in archive department and kept for a minimum of 5 years after the completion of the study. Your identity will not be revealed to any unauthorized persons and will be protected to the extent allowed by law. You will not be personally identified in any reports or publications that may result from this study.

Costs and Compensation

Your participation in the study will take approximately 5 minutes. You will not be paid for the study procedure. You will not be compensated for the stool and blood specimen.

Right to refuse or withdraw

If you choose to participate or withdraw from the study at any time you will still receive treatment at the health centre.

If the study design or use of the data is to be changed, you will be so informed and your consent re-obtained. You will be told of any significant new findings developed during the course of this study, which may relate to your willingness to continue participation.

Questions

If you have any questions, related to this study please contact the researcher, MURANGWA Anthere, Rwanda Military Hospital; Telephone: +250788740490/+254717134945;

If you have any question related to your rights as a participant please contact the chairperson of Rwanda Ethics Committee, Dr. Jean Baptiste MAZARATI at 0788309807

or Dr Laetitia NYIRAZINYOYE the secretary of the committee at 0738683209 and the KNH/UoN-ERC on telephone number (254-020) 2726300 Ext 44355.

My signature below indicates that I have decided to volunteer as a research subject and that I have read and I have received a copy of this consent form.

Signature (or thumb print) of participant…… Date ……

Witness…… Date……

Signature of Investigator…… Date……

Appendix 2

Consent form for research participants (Kinyarwanda version)

UBUSHAKE BUSESUYE MU GUKORANA MU BUSHAKASHATSI KUBANTU BAFITE IMYAKA 21 NO HEJURU YAYO.

Umutwe w'ubushakashatsi "Kwiga kundwara yitwa cyrptosporidiosis mu barwayi bafite ubwandu bwagakoko gatera SIDA banafite impiswi baturuka mu migi no mu byaro mu Rwanda"

Umushakashatsi

MURANGWA Anthere **Phone**: +250788406065/+254711166242

Intego z' ubushakashatsi

Mutumiwe kugira uruhare muri ububushakashatsi bufite intego yokureba uruhare rw'inzoka yomunda yitwa *Cryptosporidium* mugutera impiswi mu barwayi babana nagakoko gatera SIDA hakanarebwa ubushobozi busanzwe bwo kuyipima hakoreshejwe uburyo bugezweho bwa PCR kubitaro byatoranyijwe.

Abatumiwe kugira uruhare mu bushakashatsi

Umuntu wese ufite ubwandu bwa SIDA hamwe n'impiswi, yaba ari mubitaro cyangwa yivuza ataha.

Ibyo musabwa

Niba mwemeye kugira uruhare muri ububushakashatsi murasabwa gutanga garama 4 zumusarane mugupima iyonzoka, namiliritiro 4 z'amarasa zogukoreshwa mugupima abasirikare (CD4).

Ingaruka

Ntangaruka nimwe ihar imugutanga umusarani.

Inyungu

Muzakorerwa ibizamini byogupimwa iyonzoka hakoreshejwe uburyo bwitwa modified ZN hamwe nubwitwa PCR kugirango harebwe niba buriya buryo bwambere butanga ibisubizo byizewe.

Ibanga

Umusarane utanze uhabwa umubare wihariye, ntamazina azakoreshwa.Umwirondoro kumubare w'ibanga ubikwan'umushakashats iwenyine.Inyandiko zizava muri ubu bushakashatsi zizabikwa mu bubiko bwinyandiko mu Bitaro bya Gisirikare byu Rwanda mugihe cy'imyaka itanu.Ntamuntu numwe uzabona ibisuzo byawe atabiherewe uburenganzira nkuko biteganwa n'amategeko agenga ubushakashatsi. Ntahantu hazagaragara umwirondoro wawe haba muriraporo cyangwa muguntangaza ibyavuye muri ubu bushakashatsi.

Ikiguzi cyo kugira uruhare mubushakashatsi

Kugira uruhare muri ububushakashatsi biragufata iminota igera kuri itanu.Ntabwo uribwishyurwe kandi ntakiguzi cy'umusarane cyangwa amaraso gitangwa.

Uburenganzira bwo kwanga cyangwa kuva mubushakashatsi.

Ufite uburenganzira bwokwemera, kwanga cyangwa kuvamo igihe icyo aricyo cyose mubishakiye. Muravurwa neza nkuko biteganywa mwabamwemeye, mwanze cyangwa se muvuye muri ubu bushakashatsi.

Hagize igihinduka muri gahunda y'ububushakashatsi, muzabimenyeshwa kandi hasabwe n'ubushake bwanyu. Muzamenyeshwa icyo ababushakatsi bwagezeho.

Ibibazo

Niba hari ikibazo mufite murubu bushakashatsi mwahamagara umushakashatsi kuri tel: +250788406065/+254711166242; Niba mufite ikibazo kijyanye n'uburenganzira bwanyu butubahirejwe mwahamagara umuyobozi uhagarariye komite ishinzwe kurengera abakorerwaho ubushakashatsi mu Rwanda Dr. Jean Baptiste MAZARATI kuri telefone

0788309807 cyangwa Dr Laetitia NYIRAZINYOYE umunyamabanga wiyo Komite kuri telefone 0738683209 na KNH/UoN-ERC kuri telefone (254-020) 2726300 Ext 44355.

Umukono wanjye uvugako nasobanukiwe kandi nemeye kugira uruhare muri ubu bushakashatsi.

Umukono..…… italiki ……

Umuhamya…… italiki……..…

Umushakashatsi…… italiki…….

Appendix 3

Study questionnaire

STUDY TITLE: Prevalence of cryptosporidium infection among HIV-positive patients presenting with diarrhoea in a rural and an urban setting in Rwanda.

NB. Put a tick (√) or a cross (×) in the appropriate box (mushyire ikimenyetso√ cg ×ahabugenewe)

DATE OF INTERVIEW (Itariki) _____/_____/2014

CODE OF THE PARTICIPANT (Umubare w'ibanga): _____

Patient Location: In Patient (ari mu bitaro) ☐ **Out Patient (yivuza ataha)** ☐

SECTION A: SOCIODEMOGRAPHIC CHARACTERISTICS

Date of birth ____/____/_____(**igihe mwavukiye**)

Age of patient ☐☐ **(Imyaka**)

Where do you live? (**mutuye hehe**?)

Urban (**mu mujyi**) ☐ Rural (**icyaro)** ☐

District (**akarere**)_____Sector(**umurenge**) _____

Marital Status: (**irangamimerere**)

[1] Married ☐ **Ndubatse**

[2] Unmarried ☐ **Sinubatse**

[3] Widowed ☐ **Umupfakazi**

[4] Divorced ☐ **Baratandukanye**

[5] Others ……

Education level **(ikiciro cy'amashuri).**

Non- educated (**Ntiyize)** ☐

Primary school (**Amashuriabanza)** ☐

Secondary School (**Amashuri yisumbuye)** ☐

Mid- level colleges ☐ **Icyiciro kibanza cyakoleje**

University ☐ **Kaminuza**

Occupation **(icyomukora)**

Housework ☐ **Akaziko mu rugo**

Unemployed ☐ **Ntakazimfite**

Self-employed ☐ **Ndikorera**

Employed ☐ **Umukoreshwa**

Section B: RISK FACTORS (Ibishoboraguteraingoranezokwandura)

Which kind of water do you use? **Ni ayahe mazi mukoresha?**

(1) Untreated ☐ A**sukuye**

(2) Treated ☐ **Adasukuye**

Have you used to eat in restaurants, eat uncooked food and salade? **Wakundaga kugeza nubu kurira muri restora kurya ibiryo bidatetse cyangwa salade?**

Yes/**Yego** ☐ No/**Oya** ☐

Do you often fill the severe pain in the abdomen? **Ujya uribwa munda cyane kandi kenshi?**

Yes/**Yego** ☐ No/**Oya** ☐

How long do you suffer from diarrhoea? **Umaze igihe kingana gute ufite impiswi?**

3 weeks/**Ibyumweru 3** ☐ One month and above/**Ukwezi no hejuru** ☐

How long have you started the ART? **Watangiye ryari gufata imiti igabanya ubwandu bwa sida?**

Answer/**Igisubizo.....**

Have you ever stopped to take ARV while you had started taking them? **Waba warigeze uhagarika gufata imiti igabanya ubwandu bwa sida wari warayitangiye?**

Answer/**Igisubizo,** Yes/ **Yego**No/**Oya** ☐ ☐

How long/Igihe kinganiki.....

NB: If yes cell phone number, niba ari yego mwandike nomero ya telephone yanyu.....

THANK YOU

MURAKOZE

Appendix 4

Data collection tool

Code of participant:……..

Date of sample collection __dd___mm____yy

Date and time of arrival to the lab __dd___mm_____yy; Time_____

Modified ZN

Positive ☐

Negative ☐

PCR

Positive ☐

Negative ☐

CD4 cells number ☐

Appendix 5

Formal Ether Concentration Technique

Using a rod or stick, emulsify a pea size (1g) of stool in 7 ml of formal saline.

Sieve the emulsify faecesthro' 4-layer gauze, collecting the sieved suspension in a test-tube.

To filter add 3ml of ether to top up the volume to 10ml.

Put a stopper and shake vigorously for 1 minute.

Loosen the stopper (considerable pressure will have built up inside the tube)

Centrifuge at 2000rpm for 2 minutes.

Discard the supernatant and examine the deposit.

Appendix 6

Modified Ziehl-Neelsen staining technique for Cryptosporidium species

1. Prepare a thin smear of a fresh faecal specimen.
2. Air dry
3. Fix the smear in absolute methanol for 3 min
4. Stain the smear with cold carbol fuschin for 5-10 min
5. Wash off the stain with clean tap water
6. Decolorize with 1% acid alcohol
7. Rinse off the decolourizer with clean tap water
8. Counter stain with 0.25%w/v malachite green for about 30 sec
9. Wash off the stain with clean tap water
10. Air dry the slide
11. Examine under ×100

Appendix 7

Rwandan –ERC

approval

REPUBLIC OF RWANDA/REPUBLIQUE DU RWANDA

NATIONAL ETHICS COMMITTEE / COMITE NATIONAL D'ETHIQUE

Telephone: (250)2 55 10 78 84

E-mail: info@rnecrwanda.org

Web site: www.rnecrwanda.org

Ministry of Health
P.O. Box. 84
Kigali, Rwanda.

FWA Assurance No. 00001973
IRB 00001497 of IORG0001100

October 20, 2016

MURANGWA Anthere
Principal Investigator
(A student)

Review Approval Notice: No. 896/ RNEC/ 2016

RE: Annual Renewal of the study entitled **"STUDY ON CRYPTOSPORIDIOSIS AMONG HIV-POSITIVE PATIENTS PRESENTING WITH DIARRHEA IN A RURAL AND AN URBAN SETTING IN RWANDA"**

After reviewing your protocol by expedited review procedure of 20 October 2016, **we hereby provide Continuation of approval for the above mentioned protocol**.

Please note that approval of the protocol and consent form is valid for **12 months**.
You are responsible for fulfilling the following requirements:

1. Changes, amendments, and addenda to the protocol or consent form must be submitted to the committee for review and approval, prior to activation of the changes.
2. Only approved consent forms are to be used in the enrolment of participants
3. All consent forms signed by subjects should be retained on file. The RNEC may conduct audits of all study records, and consent documentation may be part of such audits.

4. A continuing review application must be submitted to the RNEC in a timely fashion and before expiry of this approval.
5. Failure to submit a continuing review application will result in termination of the study.
6. Notify the Rwanda National Ethics committee once the study is finished

Date of Approval: October 20, 2016
Expiration date: October 19, 2017

Sincerely,

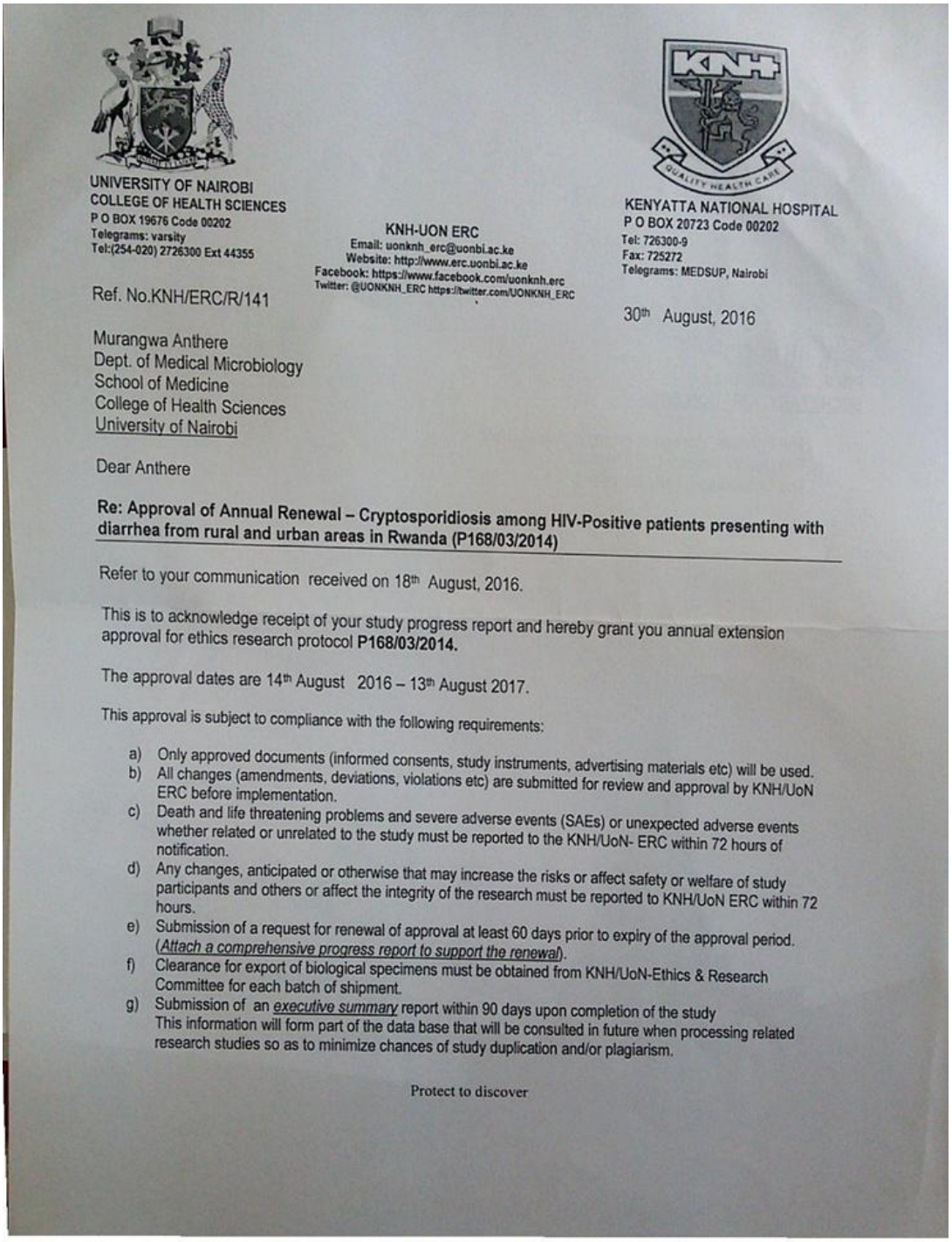
RWANDA NATIONAL ETHICS COMMITTEE
R.N.E.C
P.O. BOX:84 KIGALI

Dr. Jean-Baptiste MAZARATI
Chairperson, Rwanda National Ethics Committee.

C.C.
- Hon. Minister of Health.
- The Permanent Secretary, Ministry of Health.

Appendix 8

KNH-UoN ERC approval



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Ref. No.KNH/ERC/R/141

30th August, 2016

Murangwa Anthere
Dept. of Medical Microbiology
School of Medicine
College of Health Sciences
University of Nairobi

Dear Anthere

Re: Approval of Annual Renewal – Cryptosporidiosis among HIV-Positive patients presenting with diarrhea from rural and urban areas in Rwanda (P168/03/2014)

Refer to your communication received on 18th August, 2016.

This is to acknowledge receipt of your study progress report and hereby grant you annual extension approval for ethics research protocol **P168/03/2014.**

The approval dates are 14th August 2016 – 13th August 2017.

This approval is subject to compliance with the following requirements:

a) Only approved documents (informed consents, study instruments, advertising materials etc) will be used.
b) All changes (amendments, deviations, violations etc) are submitted for review and approval by KNH/UoN ERC before implementation.
c) Death and life threatening problems and severe adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the KNH/UoN- ERC within 72 hours of notification.
d) Any changes, anticipated or otherwise that may increase the risks or affect safety or welfare of study participants and others or affect the integrity of the research must be reported to KNH/UoN ERC within 72 hours.
e) Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. (*Attach a comprehensive progress report to support the renewal*).
f) Clearance for export of biological specimens must be obtained from KNH/UoN-Ethics & Research Committee for each batch of shipment.
g) Submission of an *executive summary* report within 90 days upon completion of the study
This information will form part of the data base that will be consulted in future when processing related research studies so as to minimize chances of study duplication and/or plagiarism.

Protect to discover

For more details consult the KNH- UoN ERC website **http://www.erc.uonbi.ac.ke**

Yours sincerely,

PROF. M.L. CHINDIA
SECRETARY, KNH-UON ERC

c.c. The Principal, College of Health Sciences, UoN
The Deputy Director CS, KNH
The Chairperson, KNH-UoN ERC