ADDIS ABABA UNIVERSITY
COLLEGE OF HEALTH SCIENCES, SCHOOL OF MEDICINE
DEPARTMENT OF MEDICAL MICROBIOLOGY, IMMUNOLOGY AND PARASITOLOGY

TITLE: PREVALENCE OF DIARRHOGENIC INTESTINAL COCCIDIAN PARASITE AND ASSOCIATED RISK FACTORS AMONG HIV INFECTED PATIENTS ON ART IN ASELLA AND ADAMA TEACHING HOSPITALS, ETHIOPIA

BY: DAGAGA KENEA (BSC)

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Table of Contents

Contents **Page**

List of Tables

Page

List of Figures

Page

ACRONOMYS AND ABREVATIONS

AAU:	Addis Ababa University
AIDS:	Acquired Immune Deficiency Syndrome
ART:	Anti- Retroviral Therapy
CD4+:	Cluster of Differentiation
CI:	Confidence Interval
ELISA:	Enzyme Linked Immuno-Sorbent Assay
HAART:	Highly Active Anti-Retroviral Therapy
HIV:	Human Immunodeficiency Virus
NSTC:	National Science and Technology Council
OPD:	Out Patient Department
RNA:	Ribonucleic Acid
SPSS	Statistical Packages for Social Sciences
UNAIDS:	United Nations Program on AIDS/HIV
WHO:	World Health Organization
ZN:	Zeihl Neelsen

ABSTRACT

Background: Intestinal coccidian parasitic infections are one of the major causes for diarrheal diseases in HIV-positive patients particularly for those with low CD4 counts. However, oocysts of intestinal coccidian parasites are mostly miss-diagnosed and under-reported as the routinely practiced wet mount stool examination is not able to detect them Investigation with coccidian staining techniques may allow correct diagnosis and treatment that may help to prevent complications.

Objectives: The objective of this study was to determine the prevalence of intestinal coccidian parasites and associated risk factors among diarrhogenic HIV positive patients on ART.

Methodology: Institutional based cross sectional study was conducted among HIV patients with diarrhea who attended the ART clinic of Asella and Adama Teaching Hospitals. During the data collection period from March 30, 2018 to August 15, 2018, Ethiopia, a total of 222 diarrhogenic HIV positive participants were included in the study. Stool samples were collected and examined in Asella Hirsch Institute of Tropical Medicine for parasites using direct smear, ParasiTrap® stool concentration technique, Modified Z-N and Auramine O staining techniques. Structured questionnaire was used to collect data on socio demographic and associated risk factors. Data was entered and analyzed using SPSS version 21 software. Logistics regressions were applied to assess any association between independent and dependent variables. Kappa value was used for method evaluation and P values < 0.05 were taken as statistically significant value.

Results: The prevalence of intestinal coccidian parasites were 22/222 (9.9%) in HIV infected patients on ART and were associated with CD4+ T-cell count <200 cells/µl [AOR, 95% CI: 10.4 (38.88, 2.8), P<0.05]. The cumulative prevalence of intestinal parasitic infection among HIV infected individuals on ART in the study populations were 18.92 % (42/222). The most prevalent parasite were *E.histolytica* (6.75%), followed by *cryptosporodium* species *and Isospora belli* (4.95%),*G.lamblia* (2.25%) and *Taenia* species (0.45%).The agreement between Modified ZN and Auramine O was found to be 0.643. Taking Auramine O as gold standard, the sensitivity and specificity of Modified ZN were 50% and 100% respectively.

Conclusions: Intestinal Coccidian parasites are frequent opportunistic intestinal parasites infecting HIV infected patients. A lowered CD4 count predisposes to acquisition of these agents. Regular monitoring of CD4 counts and screening for these opportunistic agents in the HIV infected will help reduce the mortality and morbidity associated with infections by these agents.

Key words: Diarrhea, Intestinal coccidian parasite, ART, Ethiopia.

1. INTRODUCTION

1.1 Background

Intestinal parasites are major concerns in most developing countries where HIV/AIDS cases are concentrated, a region which is currently experiencing the highest burden of HIV/AIDS. In the absence of HAART, HIV/AIDS patients in developing countries, continue to suffer from the consequences of opportunistic intestinal and other intestinal parasites (Mengist *et al*., 2015).

Intestinal coccidian parasites are a group of protozoan parasites which parasitize the epithelial cells of the intestinal tract of their hosts and most infections usually produce mild, self-limiting infections in man, but they now constitute a serious public health problem, especially in developing countries with inadequate sanitary conditions coupled with widespread HIV/AIDS infection (Djieyep *et al*., 2014).

Intestinal coccidian parasites are transmitted when matured oocyst contaminated with drinking or recreational water of infected humans or cattle contact with infected persons (i.e., those in the same household or child care facility); and oral –anal sexual practices also increase the risk of *Cryptosporidium* infection. In contrast, humans are the only hosts and reservoirs for *Cystoisospora* and *Cyclospora*, which are generally spread via drinking fecally contaminated and inadequately treated water, ingesting fecally contaminated recreational water (rivers, lakes, pools) and eating food contaminated by food handlers(Hechenbleikner and McQuade,2015).

Intestinal coccidian parasites are more common in tropics and subtropics than elsewhere in the world (Ferreira Carneiro *et al*. 2000). *Cryptosporidium* species are found worldwide from Sub-Saharan Africa to the United Kingdom and Australia. *Isospora belli* is common in the tropics and subtropics and *Cyclospora* is endemic in Haiti, Peru, China, and Nepal (Ortega and Sanchez, 2010). *Cryptosporidium* is the most common parasite encountered in the immuno-compromised host, followed by *Cyclospora* and *Isospora (Marcos and Gotuzzo, 2013).*

In Africa, Investigations for intestinal parasitic infections in AIDS patients have focused on patients with diarrhea. *Isospora* and *Cryptosporidium* have been found consistently in such patients at prevalence rates varying from 8% to 32% and 12% to 19%, respectively, among

patients with chronic diarrhea (Henry *et al*., 1986, Colebunders *et al*., 1988, Conlon *et al*., 1990, Hunter *et al*., 1992).

In Ethiopia, intestinal parasitic diseases are among the ten top causes of morbidity nationwide (EPHI, 2008).There are different reports that showed high magnitude of intestinal parasites among people living with HIV/AIDS were reported frequently in different studies (Alemu *et al*., 2011, Gedle *et al*.,2015, Fisseha *et al*., 2017).

The oocyst of the intestinal coccidian are more frequently seen in the stool of both symptomatic and asymptomatic HIV patients (Amatya *et al*., 2011).The most widely used methods are those that enable visualization of oocysts in feces without determining the species involved and using staining techniques of fecal smears on glass slides based on the acid-resistance property of coccidian oocysts (Lindsay *et al*., 1997, Magi *et al*., 2006, Hanscheid *et al.*, 2008).

Before staining fecal smears, concentration methods are strongly recommended for the diagnosis of intestinal coccidian parasitic infections (Weber *et al*.,1991, Clavel *et al*., 1996, Kar *et al.,2011)*.After stool concentration, fecal smears can be stained by a wide variety of techniques, although the variants of Modified ZN (Henriksen and Pohlenz,1981) and Auramine-phenol are the most frequently used either in routine or research laboratories(Lindsay *et al*., 1997, Magi *et al.*, 2006, Hanscheid *et al*., 2008).

Other diagnostic method includes molecular diagnostic techniques using the Polymerase Chain Reaction to amplify 18S rRNA gene in the organism, immunological techniques such as enzyme immunoassays and serologic diagnosis (Ghaffari and Kalantari, 2014). Studies have shown the molecular diagnostic method to be more sensitive and specific than other methods (Ghaffari and Kalantari, 2014) but it is also the most expensive and widely not available in this part of the world. The immunoassay like ELISA is more sensitive than microscopy methods (Elgun and Koltas, 2011).

1.2. Statement of the Problem

Intestinal parasitic infections of the gastrointestinal tract are one of the major causes of morbidity and mortality in HIV positive individual's worldwide. Amongst these, the intestinal coccidian parasites are often implicated for protracted diarrhea in specific groups such as imunocompromised individuals (Faidah *et al*., 2016).

Despite there are large number of diarrheic HIV/AIDS patients and the diversity of existing parasitological methods for detection of intestinal coccidian parasites in stools, the current prevalence of intestinal coccidian parasites and associated risk factors among HIV infected patients in Asella and Adama Teaching Hospitals are underestimated because routinely not all HIV-infected patients are examined for the presence of this intestinal coccidian infection.

This initiated us to conduct a research on intestinal coccidian parasite among HIV infected patients by Auramine O which is more sensitive and specific than Modified Ziehl–Neelsen staining and trichrome techniques for detection of intestinal coccidian parasites (Joseph and Popoola, 2017).

Intestinal coccidian parasite like *Cryptosporidium* species, *Isospora belli, and Cyclospora* species are the most common enteric parasites in immunocompromised patients that usually lead to lethal severe diarrhea are considered as causative agents of a mild and self-limiting gastrointestinal disorders in immunocompetent individuals (Gupta*et al.,2008);* and causes for cryptosporidiosis, isosporiasis, and cyclosporiasis, respectively(Ortega and Sanchez, 2010).

Intestinal coccidian parasitic infections usually occur late in the course of HIV infection when CD4+ T-cell count is severely depleted (mostly below 200 cells/mm^3 (UNAIDS,2004).In the absence of a vaccine and affordable HAART, people living with HIV/AIDS, especially in developing countries, remain at risk of opportunistic intestinal infections (Girma *et al*., 2014).

Cryptosporidiosis caused by *Cryptosporidium* species is self-limited, but leads to persistent diarrhea in the advanced stage of AIDS and there is no effective treatment available for it (Schär *et al*., 2016). *Isospora belli* causes chronic diarrhea in AIDS patient, but can be treated effectively with available antimicrobials (Shah *et al*., 2003).

Contact with animals was an important risk factor for opportunistic intestinal parasites (Alemsegede *et al*., 2015). Drinking fecal contaminated water and eating food also are risk factors for *Isosporiasis* and *cyclosporiasis*. HIV positive patients who had owned cats, dogs and contact with farm animals especially calves, swimming in public swimming pool are also risk factors of cryptosporidiosis (Goodgame, 1996, Hunter, 2003).

African is the most affected region, with 25.6 million people living with HIV and accounts for almost two thirds of the global total of new HIV infections (WHO, 2016) especially, Sub Saharan Africa has the highest burden of HIV with an estimated 23.5 million cases are associated with intestinal protozoan parasites including *Cryptosporidium* species, *Cyclospora cayetanensis* and *Isospora belli* (WHO, 2012).

In Ethiopia, even though the prevalence of opportunistic parasites is expected to be high due to poor living standard, some published data showed low prevalence's among the general population. The available information suggests that infection with opportunistic intestinal parasites such as *Cryptosporidium*, *Isospora* and *Cyclospora* are common in HIV positive individuals from different parts of the country (Endeshaw, 2005, Adamu *et al*., 2010).

1. 3. Significance of the Study

Ethiopia is among the sub-Saharan countries with overlapping high rate of HIV and parasitic infections. Intestinal parasites are highly prevalent in Ethiopia due to shortage of clean water, lack of sewage system and other unhygienic factors that increase the probability of infection. However, only a few studies have reported the magnitude of intestinal coccidian parasitic infections among HIV infected patients on ART. The present study is, therefore, aimed to determine the prevalence of intestinal coccidian parasites in HIV infected patients.

Therefore, this study was designed to:

- Generate baseline epidemiological information.
- Generate the necessary data for planning and evaluation of ART service and
- Compare diagnostic methods for intestinal coccidian parasite detections in diarrhogenic HIV infected patients on ART in Asella and Adama Teaching Hospitals
- To predict risk for communities under consideration.

2. LITERATURE REVIEW

2.1. Intestinal Coccidian Parasites

The intestinal coccidian parasites are a group of obligate tissue parasites within the subphylum sporozoa. From intestinal protozoa parasitic infections *cryptosporidium, Isospora* and *cyclospora* species are one of the major concerns for public health, especially with increasing rate of immunodeficiency disorders (Ahmed and Chowdhary, 2015; Gupta *et al*., 2008). *Cryptosporidium* species and *Isospora belli* are the most prevalent gastrointestinal parasitic protozoan's that infect a broad range of individuals, particularly those patients who have a suppressed or deficient immunity system (Sangani*et al*., 2016).

The genus Cryptosporidium was first described in 1910 by Tyzzer (Fayer, 2010) who in 1912 also described *Cryptosporidium parvum* in the small intestine of mice (Fayer, 2010) for several decades; human cryptosporidiosis was considered a benign self-resolving infection that was caused by *Cryptosporodium parvum* (Current *et al.*, 1983). This parasite was considered to have the potential to infect a broad range of mammalian species.

With the advent of the human immunodeficiency virus (HIV)/AIDS epidemic, cryptosporidiosis became an important infection, where immunocompromised patients developed no retractable life-threatening diarrhea (Jonas *et al*., 1983, Malebranche *et al*., 1983).

Cryptosporodium parvum infects man, cattle, sheep, goats, deer, horses, buffaloes, cats and other non-mammalian vertebrates, and is accepted to be a zoonotic. The ingested form of the parasite is the oocyst (Adal, 1994) and predominantly infects the small intestine, but the parasite can also be found in the colon and the biliary tract. In the small intestine, encystations of the oocyst occur and sporozoites are released. The sporozoites penetrate enterocytes, where they develop into trophozoites that occupy an intracellular but extra cytoplasmic location and causes the pathology associated with *Cryptosporodium parvum* infection (Mannheimer and Soave, 1994).

It is now recognized as a substantial threat to HIV-infected individuals who have a lifetime risk of *Cryptosporodium parvum* infection of around 10%, but it is also responsible for substantial outbreaks of water-borne diarrhea in healthy individuals, and for diarrhea in travellers and in

children (prevalence 1-3% in the industrialized world and 4–17% in developing countries (Blackman *et al*. ,1997).

Isospora belli is monoxenous parasite, affecting human as a single host. Infection with *I. belli* is relatively uncommon in healthy individuals; but its role as an opportunistic agent in patients with AIDS has confirmed its importance as a human pathogen (Sorvillo *et al*., 1995).

Among intestinal coccidian parasites *Isospora belli* and *Cryptosporodium* species are major cause of chronic or acute diarrhea and other life threatening symptoms in HIV/AIDS patients particularly those with low CD4 counts. The infectious forms in both parasites are the oocysts, which release invasive sporozoites that enable the parasite to take up an intracellular location in the intestinal epithelial cells. The oocyst in the upper small intestine invade the mucosa, causing destruction of the brush border (Siripanth *et al*., 2004).

Isospora belli is one of the opportunistic coccidian parasites that affects HIV+/AIDS patients, especially in developing countries of Africa, Asia, and Latin America with low levels of hygiene. It is always considered as a neglected parasite and the presence of this parasite because of the lack of enough investigation, particularly in immunocompromised patients, is under estimated *(Certad et al., 2003).*

Cyclosporiasis is intestinal coccidian parasitic infection caused by *Cyclospora cayetanensis and* was described in 1993 (Ortega *et al.*, 1993). It is recognized as an important cause of food-borne outbreaks, both in the United States as well as in other industrialized nations (Ashford, 1997; Herwaldt *et al.,* 1997).

2.2. Taxonomic Classifications

Cryptosporodium species is classified into the Empire Eukaryota, Kingdom Protozoa, Phylum Apicomplexa, Class Coccidea, Order Eucoccidiorida, Family Cryptosporidiidae, Genus Cryptosporidium while *I. belli* is categorized in to the Empire Eukaryota, Kingdom Protozoa, Phylum Apicomplexa, Class Coccidea, Order Eucoccidiorida, Family Eimeriidae and Genus Isospora (Betz*et al.*, 2005).

Cyclospora cayetanensis belongs to; Super group: Chromalveolata, Sub-Phylum: Apicomplexa, Class: Conoidasida, Order: Eucoccidiorida, Family: Eimeriidae, Genus: Cyclospora Today, C.

cayetanensis is one of 19 currently recognized species of *Cyclospora* (Lainson, 2005, Lalonde and Gajadhar, 2008).

2.3. Life Cycle of Intestinal Coccidian Parasites

Oocysts of *Cryptosporidium* species, the environmentally resistant transmission stage of the parasite, are shed by infected hosts with their faeces and are immediately infectious. They may remain in the environment for very long periods without loss of infectivity: a very robust oocyst wall protects the sporozoites inside against physical and chemical damage. When a new host ingests an oocyst, the body temperature and the interaction with stomach acid and bile salts trigger encystations opening of the suture in the oocyst wall. Four motile sporozoites are released, which infect the epithelial cells of the small intestine, mainly in the jejunum and ileum. The parasite infects the apex of the epithelial cells, residing beneath the cell membrane but outside the cytoplasm. The sporozoites undergo several transformations in an asexual (merogony) and a sexual (gametogony) reproduction cycle; it is the latter that generates oocysts (Figure 1). Thin-walled oocysts may excyst within the same host and start a new life cycle (autoinfection). This can lead to heavily infected intestinal epithelia and result in malabsorptive or secretory diarrhea. Thick-walled oocysts are excreted with the faeces and ingested by a new host; life cycle continues (Velásquez et al., 2001). Oocysts of *C. parvum* are spherical, with a diameter of 4–6mm, and may be either thick- or thin-walled oocytes (Khan, 2008) .

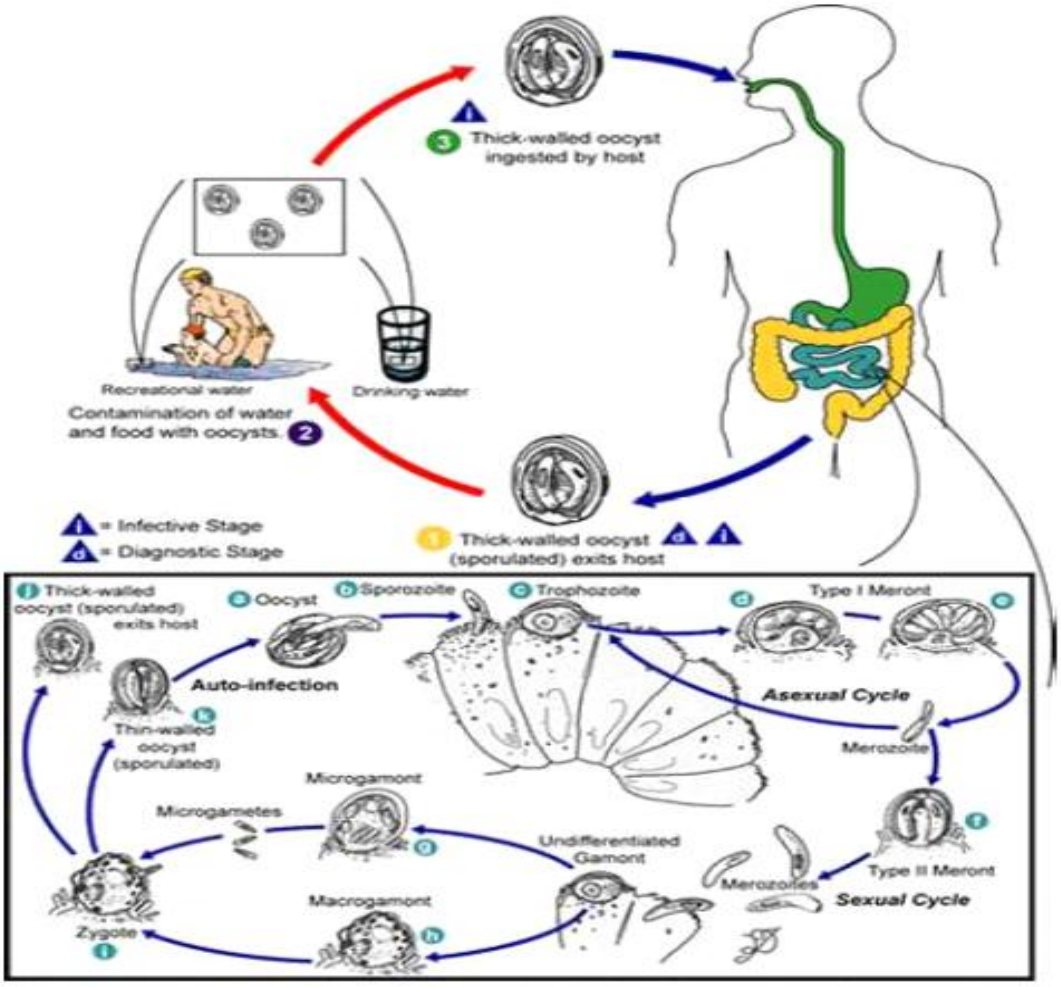

Figure 1: Life cycle of *Cryptosporidium* species (Source: CDC, 2017).

Isospora belli can be acquired by the ingestion of sporulated oocysts found in contaminated food or water. Schizogonic and saprogenic stages in the life cycle of *I. belli* have been found in human intestinal mucosal biopsy specimens. Development in the intestine usually occurs within the epithelial cells of the distal duodenum and proximal jejunum. Stages of both asexual (trophozoite, schizont and merozoite) and sexual (macrogametocyte) phases of the lifecycle of the parasite occur in the epithelium, always enclosed within parasitophorous vacuole. Eventually, oocysts are passed in the stool; they are long and oval and each contains only one immature sporont, but two may be present. Most oocyts of isospora belli excreted unsporulated, undergo a developmental period (sporulation) outside the host, and become infectious (CDC,2017).

Continued development occurs outside the body with the development of two mature sporocysts, each containing four sporozoites, which can be recovered from fecal specimen. The sporulated oocyst is the infective stage that excysts in the small intestine, releasing the sporozoites, which penetrate the mucosal cells and initiate the life cycle (figure 2). The life cycle stages, schizonts, merozoite, gametocytes, gametes and oocysts are structurally similar to those seen in the other coccidian. The oocyst stage is 23-26 by 12-17 μm and is much larger than the oocysts of related coccidian species such as *C. parvum* and *Cyclospora cayetanensis*. Sporulated oocyst of *Isospora belli* can survive for a year in the environment (CDC, 2017).

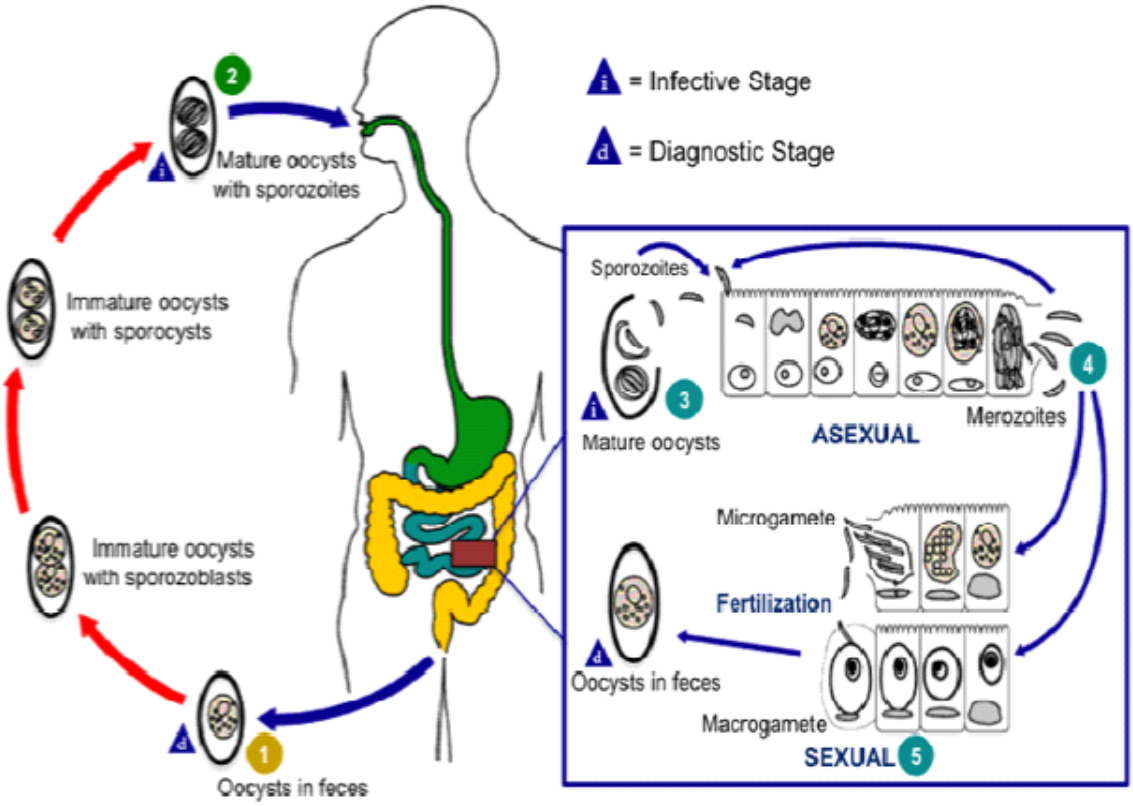

Figure 2: Life cycle of *cystoisospora belli* (Source: CDC, 2017).

Cyclosporiasis is caused by ingestion of sporulated oocysts. Sexual and asexual formas develop within an apical intra cytoplasmic parasitophorous vacuole in enterocytes of the small intestine. At the time of excretion in stool, the oocyst is immature and usually contains just one sporoblast (sometimes, two)[1]. During further maturation after excretion, the sporoblast divides in two (the oocyst now contains two sporoblasts); the sporoblasts secrete a cyst wall, thus becoming sporocysts; and the sporocysts divide twice, resulting in four sporozoites per each of two sporocysts[2]. Infection occurs by ingestion of mature (fully sporulated) oocysts: the sporocysts excyst in the small intestine and release their sporozoites, which invade the epithelial cells and initiate schizogony[3]. Upon rupture of the schizonts, merozoites are released, which invade epithelial cells and continue the cycle of asexual multiplication[4] Trophozoites develop into schizonts, which contain multiple merozoites. After a minimum of one week, the sexual stage begins, with the development of male and female gametocytes[5] Fertilization results in the development of oocysts, which are excreted in the stool[1]

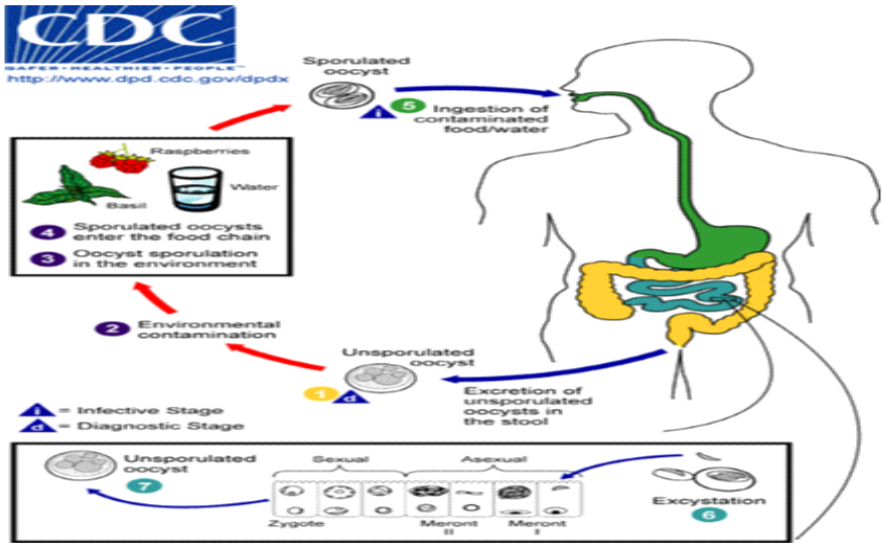

Figure 3: Life cycle *cyclospora cayatenensis* (CDC, 2017).

2.4. Epidemiology of intestinal coccidian parasite

Both cryptosporidiosis and isosporiasis have been reported worldwide. But Isospora belli Infection is more common in tropical and subtropical regions, especially Haiti, Mexico, Brazil, El Salvador, tropical Africa, the Middle East and South East Asia. Whereas cryptosporidiosis is found in numerous countries, and on all continents except Antarctica (Aliyu *et al.*, 2007).

In stool surveys of patients with gastroenteritis, the reported prevalence of Cryptosporidium are 1–4% in Europe and North America and 3–20% in Africa, Asia, Australia, and South and Central America. High rates of asymptomatic carriage (10–30%) are common in non-industrialized countries. Sero-prevalence rates are generally higher than faecal carriage rates, from 25–35% in industrialized countries to 95% in South America, increase with age and are relatively high in dairy farmers and day-care center attendants (Lewden *et al*., 2005).

In developed countries, immigrants are suspected as introducers of the isosporiasis. Chronic infections are developed in some patients and oocysts are excreted for a long duration, several months to years. This information has important implication in the era of HIV/AIDS pandemic (Takada *et al*., 2005).

Cyclospora infections have been reported in several areas of the world. It is endemic mainly in non-industrialized nations, whereas sporadic reports associated with outbreaks have been

frequently reported in industrialized countries (Sterling *et al*., 1998). In endemic settings, cyclosporiasis is more frequent in children between the ages of 2 and 5 years, showing a marked seasonal pattern (Bern *et al*., 2002). For example, in Nepal, there were higher rates of infection during the summer and rainy seasons whereas in Peru, most cases were detected between December and May, which are warmer months but without rain (Sherchand and Cross, 2001).

2.5. Pathogenesis of Intestinal Coccidian Parasite

Severe, life-threatening cryptosporidiosis and isosporiasis are usually only associated with Infants and children or individuals with poor immune systems such as elderly individuals, AIDS and leukemia patients. They can also cause asymptomatic self-limiting infections in immune competent individuals (Siripanth *et al*., 2004).

The main site of Cryptosporidium infection is the small intestine, although infection may spread throughout the gastrointestinal tract and extra-intestinal sites. In HIV patients, proximal small intestinal infections generally cause more severe diarrhea and reduced survival rates, compared to heavy infection of the colon, which, in the absence of small bowel infection, may result in asymptomatic infection or intermittent diarrhea. This group of high-risk patients frequently experience chronic or intractable disease. The whole gastrointestinal tract, including the gall bladder, pancreatic duct and even the bronchial tree can be affected. Ultrasonic examination of AIDS patients with biliary cryptosporidiosis has revealed a generalized dilation of the bile duct and gall bladder. Esophageal cryptosporidiosis, with parasites attached to the squamous mucosa and the luminal borders of sub mucosal gland and ducts has been described both in adults and in children with AIDS (Benson *et al*., 2009).

Infection and diarrhea can be caused by as few as 10 oocysts and different isolates may display different levels of virulence. The pre-patent period can be as little as a few days, but 7–10 days seems to be around the average. Oocyst shedding usually occurs within 1 week and may last for a few days to over 1 week. Diarrhea has been reported to last from 6 to 223 hrs, with averages in the 3–4 days range (Motta and Silva, 2002).

Intestinal infection with *I. belli* can cause marked villous atrophy in the small intestine. Inflammatory infiltrates in the lamina propria include eosinophils, neutrophils, lymphocytes and plasma cells. The precise mechanism causing these changes is unknown, but they result in

malabsorption. Infection of the biliary tract by *I. belli* is also possible. The parasite can complete its life cycle in the biliary tract and oocysts can be observed in bile (Cacciò *et al*., 2005). Endoscopic biopsy evaluation of patients with AIDS who had unexplained chronic diarrhea revealed an occult infection in half of the cases. However, villus and crypt changes in advanced HIV infection were independent of diarrhea or enteric infection and did not correlate with AIDS enteropathy. Subnormal epithelial proliferation in response to injury could be a factor, but the underlying cause of the changes may be due to multiple factors (Benson *et al*., 2009)

Isospora belli has also been documented as causing chronic diarrhea and a calculous cholecystitis in patients with AIDS. Although no structural means of differentiating *Isospora* to the species level are available at the light or electron microscopy levels, the unizoite cysts seen are probably part of the cycle of *I. belli*, rather than other species that could be pathogenic in immunocompromised individuals (DeHovitz *et al*., 1986)

Cyclospora cayetanensis is an important emerging infectious disease agent found worldwide causing cyclosporiasis; a condition presenting with prolonged and profuse diarrhea in immunocompetent and immunocompromised (such as AIDS) patients. It is endemic in some tropical regions of the world and mostly associated with travel to these areas (Sajjad Raja and Schelenz, 2010).

Any studies supposed that the pathogenesis is not due to invasive or toxic mechanisms. However two studies suggested that the toxin production theory is the most probable theory that causes gastrointestinal symptoms specially diarrhea and only one of them identified these toxins (Ortega and Sanchez, 2010). This theory explained that the epithelial cells invaded with sporozoites release cytokines as interleukin-8 (IL-8) that activate phagocytes to release mediators as histamine, serotonin and adenosine that increase intestinal secretion of chloride and water and inhibit absorption. Parasite invasion, multiplication or extrusion causes activation of T-cells and B-cells with release of proteases and oxidants affecting epithelial cell growth with resultant villous atrophy with crypt hyperplasia (Wang *et al*., 2002). Both cellular and humoral immune responses are included in pathogenesis so diarrhea may be prolonged in persons with poor immunological status (Connor *et al.*, 1999). Abnormal findings on lactulose or mannitol studies or studies of both have demonstrated intestinal barrier disruption. Also, abnormal findings on D-xylose studies have demonstrated malabsorption and so proximal small intestinal

involvement (Allam *et al*., 2004). These organisms can increase the contractility of jejunal longitudinal smooth muscle and decrease the basal tension with stretch of circular muscles (Connor *et al*., 1993).

2.6. Transmission

Transmission of cryptosporidiosis and isosporiasis occurs mainly by ingestion of infective oocysts through mainly faecal-oral route of contamination, by human-to-human, animal-to-human or environmentally such as in water-borne outbreaks. Nosocomial transmission has also been described (Sorvillo *et al*., 1995) .In different parts of the world. *C.parvum* has reached the public health domain when it became widely recognized as the most serious and difficult to control cause of water-borne diarrhea. This was confirmed by the major outbreak in Milwaukee, Wisconsin in 1993. Cryptosporidiosis infection in humans has been identified in more than 60 countries in six continents (Sorvillo *et al*., 1995).

Unsporulated *Cyclospora cayetanensis* oocysts are non-infectious and so direct (person-to-person) transmission through faecal exposure is unlikely to occur and food or water contaminated with freshly excreted oocysts shortly before consumption should not cause illness (Mansfield and Gajadhar, 2004).Indirect transmission can occur if an infected person contaminates the environment and oocysts have sufficient time, under appropriate conditions, to become infectious (Shields *et al., 2013)*. The oocysts are highly resistant and can still for long periods in the environment, maintaining their infectivity even under harsh environmental conditions causing the seasonality of cyclosporiasis in endemic areas (Herwaldt and Beach, 1999).

2.7. Clinical Symptom of Intestinal Coccidian Parasite

Characteristically, patients present with profuse non-bloody, watery diarrhea along with non specific symptoms, such as abdominal pain, nausea, and sweats. The acuity and severity of diarrheal disease depends on CD4+ cell status of the patient. Immunocompetent patients usually present with acute, self-limiting symptoms whereas imunocompromised patients may have protracted and severe diarrheal disease resulting in malnutrition and weight loss. In addition, AIDS patients frequently develop extra intestinal problems including gallbladder and/or biliary tract infection (Pierce and Kirkpatrick, 2009).

The major clinical symptoms of isosporiasis and cryptosporidiosis are watery diarrhea, malabsorption and wasting syndromes. The severity of the disease depends on the immune status of the individuals. The nature of diarrhea is usually secretory or malabsorpative, voluminous, intractable, watery and often cholera-like with no blood (Siripanth *et al*., 2004).

The development of chronic isosporiasis in AIDS patients have been correlated with reduced CD4+ cell count. That is, the severity of the disease is manifested in AIDS patients usually when the CD4+ cell count is below 200cells/µl. The number of CD4+ cell count is higher in AIDS patients with diarrhea when intestinal Cryptosporidiosis and Isosporiasis are not involved. Extra-intestinal coccidiosis is particularly common in patients with cell count of less than 55cells/µl. Patients with CD4+ cell count of less than 180 cells/µl have persistent infection, while patients with CD4+ cell count greater than 200cells/µl have a transient or self-limited infection (Ochiai *et al*., 2005). The clinical presentation of *Cyclospora cayatenesis* also includes either other gastrointestinal symptoms such as nausea, vomiting, abdominal cramps and loss of appetite; or constitutional symptoms such as unintentional weight loss, fever, chills, muscle aches, joint aches, generalized body aches, headache, or fatigue (Behera *et al*., 2008) and in immune-competent hosts, mild to-moderate, self-limiting diarrhea is common, while in immune-compromised hosts severe intestinal injury and prolonged diarrhea is observed (Cegielski *et al*., 1999).

2.8. Laboratory Diagnosis

Various techniques can be used in the identification and diagnosis of intestinal coccidian parasitic infection. This include staining techniques like giemsa, Modified ZN, trichrome, Auramine phenol, modified cold Kinyoun , etc. The most commonly used staining method is the acid fast modified ZN stain (Chalmers *et al*., 2011).

Other diagnostic method includes molecular diagnostic techniques using the Polymerase Chain Reaction to amplify 18S rRNA gene in the organism, the immunological techniques such as enzyme immunoassays and serologic diagnosis (Ghaffari and Kalantari, 2014). Studies have shown the molecular diagnostic method to be more sensitive and specific than other methods but it is also the most expensive and widely not available in this part of the world (Ghaffari and Kalantari, 2014).

The immunoassay like ELISA is more sensitive than microscopy methods (Elgun and Koltas, 2011). The microscopy techniques are however more widely used in this environment, hence the need to compare the commonly used staining techniques in our locality (Elgun and Koltas, 2011).

Isospora belli is diagnosed by detection of the oocysts in stool or rarely bile samples. The oocysts are elliptical, measuring 23-36 by 12-17µm and are much larger than the oocysts of related coccidian species such as *Cryptosporidium parvum* and *Cyclospora cayetanensis*. These oocysts are shed unsporulated into the environment; thus, clinical specimens from patients will contain elliptical red structures when stained with acid fast. In environmental samples, however, the spherical sporulated forms are likely to be found. Acid-fast staining methods, with or without stool concentration, are most frequently used in clinical laboratories. Oocysts can also be observed in wet preparations or iodine stained preparations of concentrated stool specimens. Oocysts will also stain positive using the Auramine protocol commonly used to detect mycobacteria (Hanscheid *et al*., 2008).

2.9. Prevention

Several strategies can be used to minimize the potential for *Isospora* and *Cryptosporidium* Contaminations of water supplies. These include eliminating sources, containing and managing wastes and enhancing natural disinfection. These may be applied in combination with water treatment to ensure that water is safe to drink (WHO, 2016). Scrupulous hand washing can reduce the risk for diarrhea in HIV-infected persons, including diarrhea caused by *Isospora* and *Cryptosporidium*. Therefore, HIV-infected persons should be advised to wash their hands after potential contact with human faeces (including after diapering small children) and after the following activities: handling pets or other animals, gardening or other contact with soil, before preparing food, before eating, and before and after sex (Endeshaw ,2005).

2.10. Treatments

Intestinal coccidian infections are treated with medical intervention. Treatment is initially aimed at properly rehydrating patients and addressing any nutritional needs. Nitazoxanide is used to treat cryptosporidiosis in immunocompetent adults and pediatric patients. Patients with AIDS having very low CD4 counts (i.e., < 50 cells/µL) are less likely to respond to therapy and can progress to fulminant infection with high mortality (Rossignol *et al*.,2001).

It is imperative to optimize highly active anti retro viral therapy (HAART) in human immune deficiency virus (HIV) patients, particularly for cryptosporidiosis for which HAART is the cornerstone of therapy.Trimethoprim/sulfamethoxazole (TMPSMX) is the treatment of choice for cystoisosporiasis and cyclosporiasis (Blanshard *et al.*, 1992).

2.11. Magnitude of the Problems

As HIV spreads, it impairs the immune cascade by affecting the CD4 T-lymphocytes, causing a marked drop in their number, while simultaneously multiplying in the host cells. The disequilibrium in the resistance of the host makes the host susceptible to a wide array of diseases, both opportunistic and non-opportunistic. The gastrointestinal tract is one of the most common sites affected in HIV-positive patients, with diarrhea being the commonest manifestation.
According to research done in Tribhuvan University Teaching Hospital, Nepal on Enteric parasitic infection among HIV-infected patients on ART were 28/112(25%) and CD4 T-cell count <200 was found to be associated with opportunistic parasitic infection (OR = 3.2, 95 % CI 1.2–7.8) (Ghimire *et al*.,2016).

According to research done in New Delhi, India to assess the intestinal parasitosis in relation to Anti-retroviral therapy, CD4+ T-cell Count and diarrhea in HIV Patients. It was found that the prevalence of opportunistic parasites in HIV patients presenting diarrhea were 47% and from this 12% and 10% prevalence were *cryptosporidium* species and *Isospora belli* respectively. *Cryptosporidium* species had significantly associated with CD4<200 cells/µl (Khalil *et al*., 2015).

According to the study done in Nigeria, HIV centers in Mubi, on prevalence of intestinal coccidian parasites burden in HIV/AIDS patients on antiretroviral therapy in HIV were (77.4%) were positive for *Cryptosporidium parvum* and *Isospora belli*. There was a highly significant association between the CD4 count and prevalence coccidian parasites ($p < 0.05$) (Djieyep*et al*., 2014).

Another study done in clinic of the University of Ilorin Teaching Hospital, Nigeria, for Comparism of various Staining Techniques in the Diagnosis of Coccidian Parasitosis in HIV infection positivity rate of 55% was reported. Modified ZN when compared to Auramine

staining, had 80% sensitivity, 76.7% specificity, positive predictive value of 69.9%, and negative predictive value of 85.2% in test subjects (Joseph and Popoola, 2017).

According to study in Asella Hospital on prevalence of opportunistic parasites *cryptosporidium parvum* and *Isopora belli* patients on ART (2.6%, 5/196). There was a statistically significant association between the prevalence of *C. parvum* and *I. belli* infections and CD4 count with <200 was (56.4%) (P= 0.0001) (Raga *et al*., 2014).

Another study in Bahir Dar Referral Hospital, Ethiopia also reported that an overall prevalence of 30.6% enteric protozoan infection. A CD4+ T-cell count of <200 cells/ml were significantly associated with the overall prevalence of enteric protozoan infection. With regard to the on-ART subjects, the overall prevalence of opportunistic intestinal parasites infection was 3% (6/200) due to *Cryptosporidium* species. *Isospora belli* were not reported from patient on-ART in this study (Kiros *et al*., 2015).

Another cross sectional study was carried out in Butajira, southern Ethiopia, which also reported that the Opportunistic intestinal parasites among HIV/ AIDS patients receiving HAART were observed in 28 (8.7%) participants. The most prevalent opportunistic intestinal parasites were *C. parvum* (5.9%) and *Isospora belli* was 2.8 % (Gedle *et al*., 2017).

According to study conducted in Jimma Health Center, Ethiopia on Prevalence of opportunistic intestinal parasitic infection among HIV infected patients who are taking antiretroviral treatment were 15.38% (Zeynudin *et al*., 2013).

3. OBJECTIVES

3.1. General Objective

- To determine the prevalence of intestinal coccidian parasites and associated risk factor among diarrhogenic HIV infected patients on ART in Asella and Adama Teaching Hospitals from March 30, 2018 to August 15, 2018.

3.2. Specific Objectives

- To determine the prevalence of intestinal coccidian parasites among diarrhogenic HIV infected patients on ART in study sites.
- To compare intestinal coccidian parasites diagnostic performance of Modified ZN and Auramine O techniques.
- To identify associated risk factors for infections with intestinal coccidian parasite among diarrhogenic HIV infected patients on ART in the study sites.

4. METHODS AND MATERIALS

4.1 Study Area

The study was conducted in Asella and Adama Teaching Hospitals**.** Both referral hospitals are found in the Oromia region of southeastern Ethiopia approximately 175 and 100 kilometers from Addis Ababa, respectively, serving a catchment population of 4 million and 3.5 million people, respectively. Both hospitals provide general outpatient and inpatient services. These two hospitals are selected purposively because there are large numbers of HIV patients under follow up care as well as these hospitals provide many health care services including pediatric HIV testing, counseling. Currently, there are 8050 and 23,000 HIV positive patients registered in ART clinic of Asella and Adama Teaching Hospitals, respectively.

4.2. Study Design and Period

Institutional based cross-sectional study design was implemented to determine the prevalence of intestinal coccidian parasite infections and associated risk factors among diarrhogenic HIV infected patients on ART in Asella and Adama Teaching Hospitals from March 30, 2018 to August 15, 2018, Ethiopia**.**

4.3. Population

4.3.1. Source Population

All HIV infected patients attending ART clinic of Asella and Adama Teaching Hospitals.

4.3.2. Study population

All HIV infected patients with diarrhea attending ART clinic.

4.4. Eligibility

4.4.1 Inclusion Criteria: -

- All HIV infected patients on ART with diarrhea.

4.4.2. Exclusion Criteria

- All HIV infected patients on ART with severe illness who are unable to participate in the study.

4.5. Sample Size and Sampling Procedures

4.5.1. Sample Size

For Asella and Adama Teaching Hospitals the sample size was determined by using single population proportion formula based on the following assumptions, according to the study conducted in Felegehiwot Referral Hospital, Bahir Dar, Ethiopia the prevalence of opportunistic protozoan infections among individuals living with HIV was 7.1 % (Kiros *et al*., 2015).

$$n = \frac{Z^2 P (1-P)}{d^2}$$

$n = \frac{(1.96)^2 x (0.071)(0.929)}{(0.05)^2}$ with adding 10% of non-response rate and the total sample size is 111

The same assumptions were considered for the sample size calculation in Adama Hospital. Therefore, the total sample size was 111 +111= 222.

4.5.2. Sampling Procedure

The sample was taken by using non-random sampling technique. Using a serial sampling method, stool samples were collected from all HIV infected patients with diarrhea on ART until the desired sample size (n=222) had been reached.

4.6. Variables of the Study

4.6.1. Dependent Variables

- Prevalence of intestinal coccidian parasitic infections

4.6.2. Independent Variables

- CD4+ T cell count
- Socio-demographic characteristics
- Clinical variables and life style.

4.7. Data Collection Procedures

Site assessment and pre-test was done prior to data collection, adjustment was made accordingly. Stool samples were collected after patients and guardians got informed about the objective of the study and gave their consent. A structured questionnaire was used to assess independent variables. Before collection of stool samples, all adult and children's parents/care-takers were oriented and materials to handle stool specimens were provided by medical laboratory technicians or technologist.

4.8. Specimen Collection and Processing

4.8.1. Stool specimen collection: A single fresh fecal sample, about 7- 10ml was collected from each consenting study subject on the same day of enrolment using wide mouth screw capped sterile container. Stool samples were divided into two parts, one of which was prepared for Parasep® trap concentrated mount and fresh unpreserved portion was prepared for a direct saline. A CD4+ T cell count was taken from logbook of Asella and Adama Teaching Hospitals.

4.8.2. Microscopic Examinations

Parasitological examination was done in collected stools samples immediately for the presence of parasites. Microscopic examination was carried out by direct wet mount using normal saline (0.85%) and Parasep® trap concentrated mount. The sediment was prepared and smeared from each specimen on two glass slides and each slide stained with one of the staining procedure to be compared: the Modified ZN and Auramine O staining method.

Direct wet mount: was performed for the detection of ova, larvae, trophozoites and cysts of intestinal parasites and iodine wet mount and was immediately examined for intestinal parasites by 10X and 40X objectives under light microscope and the result was recorded for treating physicians(Cheesbrough, 2006) .

Modified ZN staining method; was performed on fecal smears to detect oocysts of intestinal coccidian parasites. Thin smear was prepared directly from sediment of concentrated stool and allowed to air dry. The slides were fixed with methanol for 1 minute and stained with carbol fuchsine for 15 minutes. After washing the slides in tap water, they were decolorized with 1% acid alcohol for 10-15 seconds and counterstained in 0.3% methylene blue for 60 seconds and then the slides were washed in tap water.

All the stained smears were screened for oocyts of *cryptosporidium*, *Isospora* and *cyclospora*. Modified acid fast stain positive oval to round structures with size varying from 2-6μm, with or without the presence of retracted cytoplasm were identified as cryptosporidium oocyst. *Cyclospora* oocysts were identified as acid-fast round structures with crumpled cellophane appearance and approximate size of 8-10μm. Similarly, pink oval structures20-30μm by 10-19μm were identified as *Isospora* oocysts under light microscope by 100X objective after air drying and systematically at least one length (approximately 100 microscopic fields) before reporting a negative results (Cheesbrough ,2006).

Auramine-O staining Method: was also performed on fecal smears to detect oocysts of intestinal coccidian parasites. Thin smear was prepared directly from sediment of concentrated diarrheic stool and allowed to air dry. Then, stained with filtered 0.1% Auramine solution and left for 20 minutes. The slides were washed and decolorized with 0.5% Acid alcohol, for 2 min .Then, the slides were washed and 0.5% potassium permanganate solution was applied for 3 minutes.

Finally, the slides were washed in tap water and air-dried. Stained smear were examined approximately 30 microscopic fields by using fluorescent microscope before reporting a negative results. Oocyst of intestinal coccidian parasites appear as green fluorescing oval and round structures of Auramine- O stain under fluorescent microscope.

4.9. Data Processing and Analysis

Quantitative data was generated from the questionnaires and the health record analysis concerning socio-demographic and other associated risk factors with the clinical findings (positivity or negativity of intestinal coccidian parasites) were entered into SPSS 21 versions statistical software for statistical analysis. Descriptive statistics was used to calculate the percentages of infection rates among the study population in relation to their demographic characteristics such as age, sex, marital status, occupation and the major risk factors.

Binary logistic regression was utilized to analyze associations between infections of intestinal coccidian parasites and their corresponding risk factors and multivariate logistic regressions was utilized to identify potential risk factors for parasitic infections. Kappa value was used for method evaluation and P-values obtained was considered to be statistically significant when less than 0.05.

4.10. Data Quality Assurance

All laboratory analyses were carried out using standard operating procedures (SOPs) (see Annex –V).

Pre-analytical: Adequate stool specimen (7-10 ml diarrheic stool) was collected using carefully labeled, dry, leak proof, grease free transparent stool caps. The specimen was kept free of contamination from water, soil, and urine. Specimen contaminated with water, urine and soil were rejected.

In Asella Teaching Hospital, stool samples were collected and examined immediately in Asella Hirsch Institute of Tropical Medicine for opportunistic and other intestinal parasites. Whereas, in Adama Teaching Hospital, all collected stool specimen were collected and examined for intestinal parasites by direct wet mount and reported for treating physician in Adama Teaching Hospital laboratory. Then, the left stool samples were stored in deep freezer ($-81^{o}C$) in Adama Teaching Hospital laboratory and transported to Asella Hirsch Institute of Tropical Medicine by cold box for detection of intestinal coccidian by Modified ZN and Auramine O techniques.

Analytical: Direct stool examination was performed within 30 minutes of collection and appropriate amount of stool sample was used to make a good smear devoid of air bubbles. The ova, larva, cyst and trophozoites of helminthes and protozoa were observed by10X and 40X objective under light microscope. The oocysts of intestinal coccidian parasites were examined under 20X and 40X objectives under florescent microscope without oil immersion for Auramine O stained film and 100 X objectives under light microscope with one drop of oil immersion and for Modified ZN stained film. Auramine O and modified ZN stained stool smears were examined for at least 30 and 100 microscopic fields respectively.

Post-analytical: All microscopic findings was encoded and reported appropriately.

4.11. Ethical considerations

The study was approved on March 20, 2018 by the Research and Ethical Review Committee of Department of Medical Microbiology, Immunology and Parasitology Addis Ababa University (Meeting Number DERC/17/18/02-N) and Permission was obtained from Asella and Adama Teaching Hospitals administrations.

The study subjects were informed about the objective of the study and all the reasons why participants were chosen and why the research was done were explained in the information sheet by local language. Informed consent was obtained from all participants before testing and commencing the study. All the information from the study participants was coded to maintain confidentiality and data was collected after informed consent signed. The positive results were timely reported to the clinicians for appropriate intervention.

4.12. Operational Definition

Diarrhea- is the passage of three or more loose or liquid stools per day (or more frequent passage than is normal for the individual) which can be caused by a variety of bacterial, viral and parasitic organisms.

Acute diarrhea- types of that last less than 2 weeks.

Chronic diarrhea- type of diarrhea lasts longer than 2 weeks.

Sensitivity- is the probability that a truly infected individual will test positive.

Specificity- is the probability that a truly uninfected individual will test negative.

Reference standard test / gold standard' test- is a test that is used to identify which subjects are truly infected and which are uninfected against which the sensitivity and specificity of tests are evaluated.

Positive predictive value (PPV) - is the probability that those testing positive by the test are truly infected.

Negative predictive value (NPV) - the probability that those testing negative by the test are truly uninfected.

True positive- the individual has the condition and tests positive for the condition

True negative- the individual does not have the condition and tests negative for the condition

False positive- the individual does not have the condition but tests positive for the condition

False negative- the individual has the condition but tests negative for the condition.

6. RESULT

6.1. Socio-demographic Characteristics of the Study Participants

A total of 222 participants were recruited into study among HIV infected patients on ART with diarrhea in Asella and Adama Teaching Hospitals. Majority of study participants were female (64.86%) and 45% were from Asella Teaching Hospital and (55%) were from Adama Teaching Hospital (Table 1).

Most of the study participants were urban residents (78.8%); out this (69.37%) and (88.28%) in both Asella and Adama Teaching Hospitals respectively. The assessment of educational status of HIV infected patients involved in the study showed (40.54%) and 59.46% were literate and illiterate respectively. Majority of study participants jobs (40.54%) were housewife. Most of study participants (67.57%) were married (Table 1).

Table 1: Socio demographic characteristics of study participants in Asella and Adama Teaching Hospitals.

Variables		Asella n (%)	Adama n (%)	Total n(%)
Sex	**Female**	50(45)	72(64.86)	122(55)
	Male	61(55)	39(35.14)	100(45)
Age	**<15**	3(2.7)	3(2.7)	6(2.7)
	15-29	26(23.42)	19(17.12)	45(20.3)
	30-44	57(51.35)	66(59.46)	123(55.4)
	>44	25(22.52)	23(20.72)	48(21.6)
Residence	**Urban**	77(69.37)	98(88.28)	175(78.8)
	Rural	34(30.63)	13(11.71)	47(21.2)
Education	**Literate**	77(69.37)	45(40.54)	122(55)
	Illiterate	34(30.63)	66(59.46)	100(45)
Occupations	**Student**	15(13.5%)	12(10.81)	27(12.2)
	Farmer	15(13.5%)	12(10.81)	27(12.2)
	Housewife	24(21.6)	45(40.54)	69(31.2)
	Government employee	11(9.9)	13(11.71)	24(10.8)
	private employee	18(16.23)	15(13.51)	33(14.9)
	Daily laborer	28(25.23)	14(12.61)	42(18.92)
Marital status	**Married**	78(70.27)	75(67.57)	153(68.92)
	Unmarried	19(17.12)	20(18.02)	39(17.57)
	widowed	9(8.12)	6(5.41)	15(6.76)
	Divorced	5(4.5)	10(9.01)	15(6.76)

n=number of study participants

6.2. Prevalence of Intestinal Parasites among Diarrhogenic HIV Infected Patients on ART.

Prevalence of total intestinal parasite was determined by Wet mount and Auramine O techniques. Intestinal coccidian parasites were detected by Modified ZN and Auramine O techniques and other intestinal parasites were detected by wet mount technique. The cumulative prevalence of intestinal parasitic infection and intestinal coccidian parasitic infections among HIV infected individuals on ART in the study populations were 18.92 % (42/222) and 9.9% (22/222) respectively. The most prevalent parasite were *E.histolytica (6.75%)* , followed by *cryptosporodium* species and *Isospora belli* (4.95%),*G.lamblia* (2.25%) and *Taenia* species (0.45%).In this case the same rate of infections of *cryptosporidium* species and *Isospora belli* were reported(Table 2).

Table.2.Prevalence of intestinal coccidian and other intestinal parasites among diarrhogenic HIV infected patients on ART in Asella and Adama Teaching Hospitals.

Type of intestinal parasite	Asella n (%)	Adama n (%)	Total n (%)
***Cryptosporidium* species**	9(8.12)	2(1.8)	11(4.95)
Isospora belli	6(5.41)	5(4.5)	11(4.95)
Entamoeba histolytica/dispar	10(9)	5(4.5)	15(6.76)
Giardia lamblia	3(2.71)	2(1.8)	5(2.25)
***Taenia* species**	1(0.9)	0(0)	1(0.45)

n.: number of positive cases

6.3. Distributions of Intestinal Coccidian Parasite in Relations to CD4 Status.

In this study, Intestinal coccidian parasitic infections status with respect to the CD4+ T-cell count was variable among the study participants and there was an association between CD4+ T-cell count and the intestinal coccidian parasite among patients on ART.The maximum intestinal coccidian parasitic infection was 15(37.5%) in patients with a CD4+ T-cell count of <200 cells/ml.

Among intestinal coccidian parasite *cryptosporidium* species and *Isospora belli* were detected. More infection with cryptosporidiosis (4.05%) and isosporiasis (2.7%) was found in a low CD4+ T-cell count (<200 cells/ml) (figure 4).

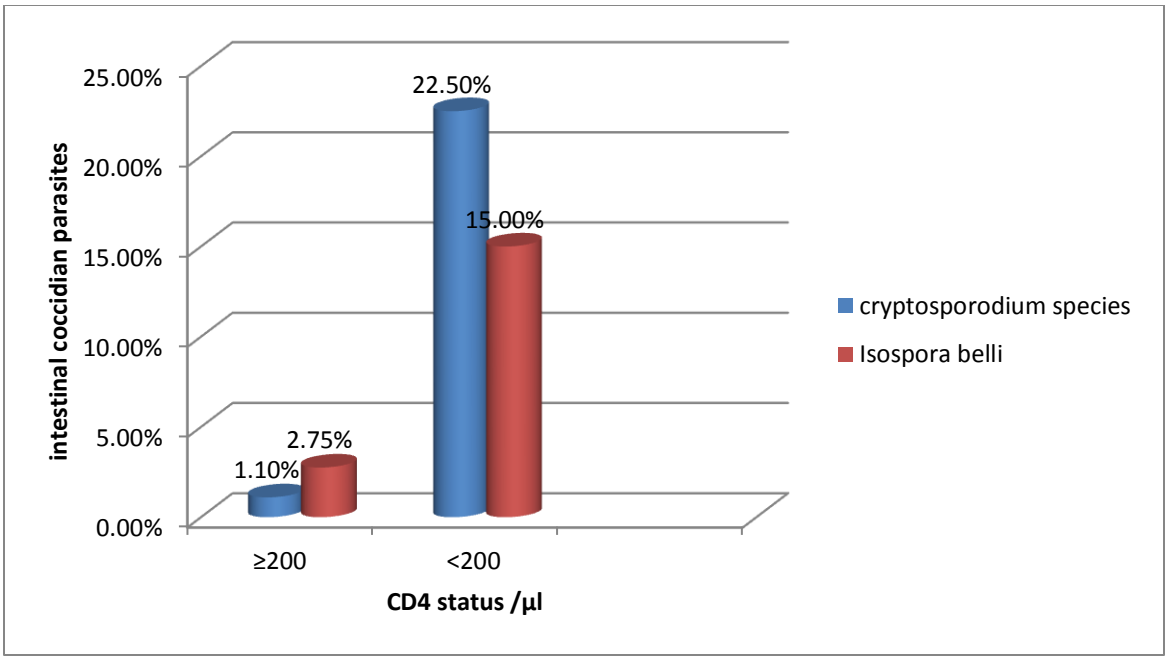

Figure 4.Status of intestinal coccidian parasite with reference CD4+ T cell count.

6.4. Diagnostic Performance of Modified Z-N and Auramine O in Diagnosis of Intestinal Coccidian Parasite.

The more sensitive one was taken as "Gold standard" to estimate sensitivity, specificity, negative predictive value and positive predictive value of each method in detecting intestinal coccidian parasitic infections. Out of the stool samples processed 4.95% (n=11) were positive intestinal coccidian parasites by modified Z-N while intestinal coccidian were 9.9% (n=22) by Auramine O staining methods.

Auramine O was found more sensitive in intestinal coccidian parasites than modified ZN technique. However, specificity and positive predictive values of detecting intestinal coccidian was parasites was similar (100%) in both tests. Study participants, who were Auramine-O positive and were not detected by Modified ZN with Measure of agreement (kappa value) (0.64) (Table 3).

Table 3: Comparison of Modified Z-N with Auramine O in detections of intestinal coccidian parasitic infections in among diarrhogenic HIV infected patients on ART in Asella and Adama Teaching Hospitals.

Test method	Result	Auramine O							Kappa value
		No +ve	No-ve	Total	Sensitivity	Specificity	PPV	NPV	
					50	100	100	94.8	0.643
Modified ZN	No +ve	11	0	11					
	No-ve	11	200	211					
	Total	22	200	222					

ZN-Ziehl-Neelsen, PPV-positive predictive value, NPV- negative predictive value, No+ve-number of positives, No-ve- number of negatives.

6.5. Univariate and Multivariate Analysis of Risk Factors Associated with Intestinal Coccidian Parasites among Diarrhogenic HIV infected Patients on ART.

In the current study based on univariate analysis residence (P=0.001), having pet animals (P=0.044), contact with cattle's excreta (0.001), diarrhea (P=0.001) and CD4 count (P=0.001) were associated with overall prevalence of opportunistic parasite among patients on ART. However, other socio demographic characteristics were not associated with prevalence intestinal coccidian parasite. The prevalence of opportunistic parasites among study subject who live in urban populations were (6.3%) whereas, in rural populations were (23.4%). There was an associations between opportunistic parasitic infection and residence 0.220(0.545, 0.088), P=0.001). Patients living in rural vicinity were 0.22 times more likely to have opportunistic parasites than urban populations.

The prevalence of opportunistic intestinal parasites among study subject having pet animals was (15.1%) and not having pet animals was (6.6%). There was an association between opportunistic intestinal parasites and having pet animals (OR 2.5 (6.2, 1.024), P=0.044). The odds of having opportunistic intestinal parasites were 2.5 times higher in HIV patients having pet animals than those not having pet animals.

High prevalence of opportunistic parasite were detected in study subject who have contact with cattle's excreta (22.2%) than those who have no contact with cattle's excreta were (6%).The

odds of having opportunistic intestinal parasites 4.5 times higher in HIV patients who have contact with cattle's excreta than not contact with cattle's excreta.

CD4 count was also associated with opportunistic intestinal parasite. The odds of patients who had CD4+ T-cell count less than 200 cells/µl were 15 times more likely to have opportunistic intestinal parasites than those CD4+ T-cells greater than or equal to 200 cells/µl.

High prevalence of opportunistic parasites were detected in study subject who have chronic diarrhea was (23.4%) and acute diarrhea was (6.3%). The odds of patients with acute diarrhea were 4.45 times not more likely to have opportunistic intestinal parasites than in those with chronic diarrhea.

In multivariate analysis using logistic regressions, only CD4+ T-cell count less than 200 cells/µl (AOR10.4 (38.88,2.8),P=0.001) was identified as significant risk factor for opportunistic intestinal parasites among HIV patients. The odds of patients who had CD4+ T-cell count less than 200 cells/µl is 10.4 times more likely to have opportunistic intestinal parasites than those CD4+ T-cells greater than or equal to 200 cells/µl (Table 4).

Table 4: Univariate and multivariate Associations of risk factors with intestinal coccidian parasites in Asella and Adama Teaching Hospitals among diarrhogenic HIV infected patients on ART.

Variables		Total Number	No.+ve (%)	OR (95% CI)			
				COR	p-value	AOR	P-Value
Sex	**Female**	122	8(6.6)	0.4(1.07,0.17)	0.07	0.614(3.1,0.12)	0.556
	Male	100	14(14.0)	1		1	
Residence	**Urban**	175	11(6.3)	0.22(0.55-0.09)	0.001	0.6(2.01,0.18)	0.406
	Rural	47	11(23.4)	1		1	
Education	**Literate**	122	11(9.0)	0.8(1.9,0.3)	0.623	0.71(2.3,0.2)	0.561
	Illiterate	100	11(11.0)	1		1	
Occupations	**Student**	27	1(3.7)	0.2(1.2,.02)	.097	0.2(2.6,0.02)	2.588
	Farmer	27	4(14.8)	0.74(2.8,0.2)	.652	0.73(4.4,0.12)	4.385
	House wife	69	5(7.2)	0.3(1.1,.101)	.070	0.6(3.61,0.90)	3.608
	Government employee	24	1(4.2)	0.2(1.6,.022)	.123	0.24(3.1,0.02)	3.065
	Private employee	33	3(9.1)	0.44(1.75,.103)	.236	1.3(8,0.204)	7.936
	Daily laborer	42	8(19.0)	1		1	
Practice of open field defecation	**Yes**	127	17(13.4)	2.3(5.82,0.9)	0.086	2.94(10.8,0.8)	0.106
	No	95	5(5.3)	1		1	
Having pet animals	**Yes**	86	13(15.1%)	2.5(6.2,1.024)	0.044	1.11(3.42,0.36)	0.860
	No	136	9(6.6%)	1		1	
Contact with cattle's excreta	**Yes**	54	12(22.2%)	4.5(11.17,1.83)	0.001	2.32(7.7,0.7)	0.170
	No	168	10(6%)	1		1	
Eating uncooked vegetables	**Yes**	127	17(13.4)	2.8(7.8,0.99)	0.053	1.67(6.35,0.45)	0.439
	No	59	5(5.3)			1	
Diarrhea	**Acute diarrhea**	175	11(6.3%)	1		0.62(2.1,0.2)	0.446
	Chronic diarrhea	47	11(23.4%)	4.56(11.32,1.83)	0.001	1	
CD4	**<200**	40	15(37.5%)	15(40.4,5.6)		10.4(38.88,2.8)	0.001*
	>=200	182	7(3.8%)	1		1	

No+ve: Number of Positive Cases, COR: Crude Odd Ratio, AOR: Adjusted Odd Ratio, CI: Confidence Interval

7. DISCUSSION

In this study, the prevalence of intestinal protozoan infections among HIV-infected patients on ART with different immune status was investigated. The examination of stool samples collected from study subjects revealed an overall prevalence of 9.9% (22/222) intestinal coccidian parasites (Table 2).

Intestinal parasites, especially the opportunistic ones are common cause of morbidity and mortality in HIV/AIDS patients. Due to down-regulation of the immune system, numerous intestinal coccidian parasites are responsible for infecting the gut of HIV-positive patients. *Cryptosporidium* species and *I. belli* are the most prevalent gastrointestinal parasitic protozoan that infect a broad range of individuals (WHO, 2004).

These infections usually occur late in the course of HIV infection when CD4+ T-cell count severely depletes (mostly below 200 cells/mm^3) and one of the most common cause of diarrhea in imunocompromised individuals (Sangani et *al*., 2016).

However, the current finding is significantly higher than previous study reported from Asella (2.6%, 5/196) (Raga *et al*., 2014), Bahir Dar (2.6%) (Kiros *et al*., 2015), and Butajira, southern Ethiopia (8.7%) (Gedle *et al*., 2017). This might be due to fact that the using sensitive laboratory technique Auramine O and including only diarrheic patients in the study. In addition to this, Patients with AIDS having very low CD4 counts (i.e., < 50 cells/µL) are less likely to respond to therapy and can progress to fulminant infection with high mortality and morbidity (Blanshard *et al*., 1992).

The prevalence of intestinal coccidian parasites were low when compared with other studies conducted in Tribhuvan University Teaching Hospital, Nepal (25%) (Ghimire *et al*., 2016), Nigeria, HIV centers in Mubi (77.4%) (Djieyep *et al*., 2014), New Delhi, India (47%) (Khalil *et al*., 2015) and in Jimma Health Center, Ethiopia (15.38%) (Zeynudin*et al*., 2013).The lower rate of infection in the current report is likely to be the outcome of improved care and treatment provided to people living with HIV/AIDS. On the other hand, this might be due to the reality that use of ART drug with better follow-up through laboratory tests, lead to a robust immunological response against infections of opportunistic parasites, especially, *Cryptosporidium* species and

Isosppora belli since these two coccidian parasites are the most prevalent causes of chronic diarrhea and other life threatening symptoms among HIV/AIDS patients.

As shown, only CD4+ T-cell count less than 200 cells/µl (AOR 10.4 (38.9, 2.8), P=0.001) was identified as significant risk factors for intestinal coccidian parasites among HIV patients. Those patients who had CD4+ T-cell count less than 200 cells/µl 10.4 times more likely to have opportunistic intestinal parasites than those CD4+ T-cells greater than or equal to 200 cells/µl. Among the study participants (n=222), 40(18%) had CD4 counts <200 cells/µl, among which 15(37.5%) were infected by intestinal coccidian parasites. This finding was similar to the finding of Asella 98/271(36.2%),Bahir Dar 38.8 % (26/67) (Rega *et al*., 2013, Kiros *et al*., 2015,) CD4+ T-cell count less than 200 cells/µl was significant risk factors for intestinal coccidian parasites among HIV patients. The significantly higher prevalence of intestinal coccidian parasites in particular *Cryptosporodium* species and *Isospora belli* infections in individuals with lower CD4+ cell count could be due to the fact that opportunistic intestinal parasites mostly affecting HIV patients and other immuno-compromised individuals with low CD4+ count (<200 cells/µl) (Ochiai *et al*.,2005).

The staining techniques compared in this study were modified ZN and Auramine O. both identified satisfactorily acid fast oocysts in stool of both HIV infected patients. The technique that required shorter time to stain and screen stained slide was the Auramine O stain. The widely used technique in this environment is the modified ZN stain. Hence we need to compare the Auramine O methods with it. This is because most centers do not have the needed fluorescent microscope required to view. The reagents used in the modified ZN staining is however readily available, affordable and cheaper compared to the reagents used in the other staining techniques. This finding is similar to the report in University of Ilorin Teaching Hospital, Nigeria who compare three staining technique in detections of intestinal coccidian parasites (Joseph and Popoola ,2017).Of these two techniques, Auramine O is more sensitive than Modified ZN staining technique. This might be due to the ease of staining, faster, less observer fatigue when reporting, less skill required when screening and reporting for the fluorescing oocysts are easily identified.

8. LIMITATIONS OF THE STUDY

Main limitations are as follows:

- Since study was cross sectional study, cause and effect relationship between opportunistic intestinal parasites and risk factors was not studied.
- Due to financial constraints sensitive techniques like PCR was not used to identify the species of Cryptosporidium
- Due to financial and time constraints three times diarrheic stool specimen per patient was not collected to increase detection rate.
- Patient restriction on ART with diarrhea, but not for patients on ART without diarrhea and with diarrhea not on ART.

9. CONCLUSION AND RECOMMENDATION

9.1. Conclusion

The prevalence of intestinal coccidian parasite among HIV infected patients on ART was considerably high. Low CD4 count was significantly associated with intestinal coccidian parasitic infections. Opportunistic parasites among ART users were still the cause of morbidity for patients came with diarrhea. However, the importance of non-opportunistic intestinal parasitic infections should not be neglected.

High proportions of opportunistic intestinal parasites (especially, *Cryptosporidium* species and *I. belli*) infections are associated with lower CD4 counts (usually <200 cells/µl) in patients on ART. Having pet animals, contact with cattle's excreta, diarrhea significantly increased the prevalence of intestinal parasites in the study area

9.2. Recommendations

Based on our results we recommend that;

- Public health measures should continue to emphasize the importance of environmental and personal hygiene like avoiding open field defecation, contact with pet animals to obtain a better quality of life for those patients.
- Stool examination including Auramine-O staining should be routinely performed in the follow-up of HIV attending ART clinic in order to improve treatment and other preventive measures.
- Finally, since a significant number of diarrheic patients were found with no parasitic etiologies, further comprehensive etiological studies for bacterial, viral, fungal and parasitic causes of diarrhea need to be conducted in the study area in the future.

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11. Annexes

Annex-I: Individual Written Consent Form: Prevalence of Intestinal coccidian parasite infections associated risk factors among diarrhogenic HIV/AIDS patients on ART in Asella and Adama Teaching hospitals, Ethiopia

My name is Dagaga Kenea. I am MSc in medical parasitology student in School of medicine department of medical microbiology, Immunology and parasitology, Addis Ababa University; conducting my M.Sc. Thesis Research. This study was used to determine prevalence of intestinal coccidian parasite and associated risk factors among people leaving with diarrhogenic HIV infected individuals on ART. The study does not cause any harm other than expensing you a few minute to give us your stool sample and fill this questionnaires. I would also like to assure you about the confidentiality of information. You have full right to reject, to participate or to interrupt the enrolment at any time. The information that you were given us is very important to meet the objective of study to determine the main risk factors for the intestinal protozoan infections among diarrhogenic HIV infected patients on ART.

Are you willing now to participate in the study? (Tick one) Agree_____ Do not agree____

Thanks!

ART unit ID_____

Signature of the participant_____

Investigator's name _____Signature_____

Checked on date_____

The outcome is (thick one): Complete _____ Incomplete_____

Other, Specify_____

Annex II: English versions of Questionnaire for Data Collection on the Prevalence of Intestinal Coccidian parasite and Associated Risk Factor diarrhogenic among HIV infected Patients on ART in Asella and Adama Teaching Hospitals, Ethiopia.

S.NO	Questions	Response	Remark
1	**Sex**	1. Male	
		2. Female	
2	**Age**		
3	**Residence**	1. Urban	
		2. Rural	
4	**Education**	1. Literate	
		2. Illiterate	
5	**Marital status?**	3. Married	
		4. Single	
		5. Widowed	
		6. Divorced	
6	**Occupation?**	1. Student	
		2. Farmer	
		3. House wife	
		4. Government employee	
		5. Private employee	
		6. Daily laborer	
		7. Others Specify…	
7	**What is your drinking water source?**	1. Tap water	
		2. Hole water	
		3. Stream/ river water	
		4. Others specify……	
8	**Do you have latrine facilities at your home?**	1. Yes	
		2. No	
9	**If there is latrine facility what type**	1. Flush toilet	
		2. Pit	
		3. Communal latrine	
		4. Other ,specify_____	
10	**Practice of open field defecations**	1. Yes 2. No	
11	**Practice of hand washing after toilet**	1. Yes 2. No	
12	**Practice of hand washing before eating**	1. Yes 2. No	
13	**Do you have pet animals such as cat,dog,goat and etc**	1. Yes 2. No	
14	**Do you have repeated contact with cattle's excreta?**	1. Yes 2. No	
15	**Do you habit of eating uncooked vegetables**	1. Yes 2. No	
16	**Diarrheal status**	1. Less than two weeks 2. Between 2 and 4 weeks 3. Longer than 4 weeks	
17	**Abdominal pain symptoms**	1. Yes	
		2. No	
	Part 3- Immunological Variables		
18	**What is your current CD4 count?**		

Laboratory result

Direct wet mount_____

Concentration_____

Modified ZN_____

Auramine O_____

Annex III: Individual Written Consent Form (Afaan Oromoo): Namoota HIV/AIDS waliin jiraatan gidduutti tatamsa'ina dhibee protozoa n dhufani fi rakkoolee dhukkuba kana fidan irratti Hospitaala mana barumsaa Asallaa fi Adaamaa, Itoophiyaa

Maqaan koo Dagaagaa Qana'aa n jedhama.Universitii Addis Ababa,mana Barnootaa saayinsii fayyaa , muummee fayyaa Microbiology,Immunology fi Parasitology tti MSc medical parasitology kanan ta'e yeroo ammaa waraqaa qorannoo eebbaa dalaguu irrattan argama. Qo'annoon kun kan godhamu namoota HIV/ AIDS Wajjin Jiraatan Gidduutti tatamsa'ina protozoa fi rakkoolee isaan fidan irratti xiinxaluuirratti. Qo'annichi miidhaa tokkoyyuu isinrratti hin fidu, daqiiqaa muraasa gaaffiiwwan kana guutuu fi naamunaa bobbaatii nuuf kennuuf fudhattaniin alatti jechudha.Odeeffannoon kun faayidaa qo'annootiin ala hin ba'u,qo'annchaaf qofa akka oolu ni mirkaneessina. Isin immoo diduufiis ta'e jiddutti dhiisuuf mirga guutuu qabdu. Odeeffannoon isin nuuf kennitan bu'aa guddan inni qabu kayyoo qo'annoo kanaa keessaa, namoota HIV/AIDS wajjiin jiraatan gidduutti karaalee dhibeewwan garagaraa tatamsa'an to'achuu keessatti faaydaa olaanaa kan isiniif qabudha.Kanaafuu qo'annoo kana keessatti hirmaachuun akka nu gargaartan kabajaan isin gaafanna.

Amma qo'anicha keessatti hirmaachuf fedhii qabduu?

Yoo fedhii horatan itti fufuu ni dandeessu.

Galatoomaa!

Mallatoo Hirmaataa/ttuu _____ Guyyaa gaaffii _____

MaqaaQo'ataa _____ Mallattoo _____

Guyyaa ilaallame _____

Bu'aa: Xumurame _____ Hin xumuramne _____ Kan biraa ibsii _____

Annex-IV: Pre-tested Questionnaires (AfaanOromoo): Namoota HIV/AIDS waliin jiraatan gidduutti tatamsa'ina dhibeewwan pirotozoa dhibee garaa kaasaa namatti fidan mana barumsaa fayyaa Hospitaala Asallaa fi Adaama.

Kutaa I: Odeeffannoo Hawaasummaa

Tartiiba lakkoofsa	Gaaffiwwan	**Deebiii**	**Yaaada**
1	Saala keessan?	1. Dhiira	
		2. Dhalaa	
2	Umriin keessan waggaa meeqa?		
3	Fuudha/Heeruma keessan?	1. Fuudhe/Heerumte	
		2. Kophaa	
		3. Narraa du'e	
		4. Hiike	
		5. Debiinhinjiru	
4	Dalagaa	1. Barataa/ttuu	
		2. Qote bulaa	
		3. Haadha manaa	
		4. Hojjetaa mootummaa	
		5. Hojjetaa dhuunfaa	
		6. Humnaan bulaa	
		7. Kan biro-------	
5	Haala barnootaa	1. kan barate	
		2. kan hin baranne	
6	Bishaan dhugaatiif oolue essaa argattaa?	1. Boombaa	
		2. bishaanboollaa	
		3. Laga	
		4. Kan biro ibsi…….	
7	Dhibeen garaa kaasaa ammam sirra tureera	1. torban lamaan gadi	
		2. torban lamaa fi isaa ol	
8	Garaa dhukkubbii qabdaa	1. eyyee	
		2. lakkii	
Q9	Mana fincaanii mijaawaa mana keessa qabdaa?	1. Eeyyee	
		2. Lakki	
10	Gosa mana fincaanii	1. mana fincaanii bishaan qabu	
		2. mana fincaani boollaa	
		3. mana fincaanii waliinii	
		4. kan biro__	
11	Utuu hin nyaatin dura dhiqachuu	1. eyyee 2. lakkii	
13	Bineeldota kan akka adurree, saree, re'ee fi kkfni qabdaa?	1. Eeyyee	
		2. Lakki	
14	Udaan bineeldota kana tuqxee beekta	1. Eeyyee 2. lakki	
15	Kuduraa fi fudaraa utuu hin bichaatin nyaattee beekta	1. Eeyyee 2. lakki	
16	Garaan si dhukkubaa	1. eyyee 2. lakki	
17	Teessissuun hangam sirra tureera	1. turban lamaa gadi 2. tarban lama hanga turban afuritti 3. turban afurii ol	
18	Lakkoofsi seelii CD4 kee yeroo ammaa meeqa?	\	

Laboratory result

1. Direct wet mount _____
2. Concentration_____
3. Modified Z-N_____
4. Auramine O_____

Annex V: Stool Specimen Processing Procedures

A. Direct wet mount

Principle: Many parasites cause disease in man. Some of these parasites are excreted in stool; they are called intestinal parasites. Intestinal parasites can be identified by examination of fresh stool samples. In stool samples we can find worms (eg. *Ascaris lumbricoides*) and segments of worms (e.g. Taenia species) visible to the eye. By microscopic examination of fresh stool samples, we can find eggs (e.g. Hookworm) and larvae of worms (e.g. *Strongyloides stercoralis*). We also find protozoa trophozoites (e.g. Amoeba) and cysts (e.g. *Cyclospora cayetanensis*). In heavy and moderate infection, a direct smear examination with normal saline and/or iodine to stain cysts is usually sufficient. For light infections, a concentration of the stool sample might be required to find helminths (worm) eggs and protozoa by microscopic examination (Cheesbrough, 2006).

Procedure

1. Place a drop of normal saline on a clean slide
2. Using a piece of stick, place a small amount of specimen, including blood and mucus in one end of the slide and cover it with a cover slide
3. First examine microscopically using 10X objectives to give good contrast and use the 40x objective to identify trophozoites of protozoa. Reporting: Report the name of the parasite found

B. ParasiTrap - System ® stool concentration technique

Principle: ParasiTrap - System ® stool concentration technique which is an enclosed, single use disposable system and employs the principal Ridley-Allen fecal concentrations technique. Cysts, oocysts, eggs, and larvae are fixed and sediment and the fecal debris is separated in a layer between the ether and the formol water. Fecal fat is dissolved in the ether

Procedure

1. The stool samples are taken by using the plunger of the processing tube I, which is attached to the screw cap.

2. The screw cap of the processing tube I is opened. If necessary, the stool suspension can be stirred with the plunger without using any additional aids: you lift the screw cap approx. 10-15 mm above the brim of the tube and insert it quickly again until it touches the brim of the. It is important not to lift the plunger above the surface of the fluid

3. The screw cap of the processing tube I and the empty plunger is removed. Add 1,5 ml Combi Medium carefully to the sample solution

4. Screw the processing tube II with the integrated filter system firmly onto the properly loaded processing tube I from above. Now the assembled system is rotated 180° to allow the sample transfer through the attached filter into the processing tube II

5. Mixed by shaker at maximum rotary speed for approx. 10-15 sec. It is enough to slightly press the upper tube with fingertips and simultaneously hold the system. After this operation already half of the stool suspension will be collected in the lower cone-shaped processing tube II. If no shaker is available, the system is shaken thoroughly by hand for approx. 30 sec.

6. The part which remains in the upper vessel is transferred into the lower tube by shaking in vertical direction - like shaking the old clinical thermometer. During the whole shaking process active filtration occurs, which further enhances the yield of parasites. This device is designed in a way, which provides an ideal relation between suspension volume precipitation rates. The suspension is left as it is for 1-2 min. The filtered suspension is centrifuged for 5 min. at max. 1500 g (picture 6, processing by small size centrifuge see below). Instead of centrifuge, leave the system few hours, until the phases have clearly separated.

7. After centrifugation four layers have been formed in the processing tube II. The processing tube I including the filter-piece is removed

8. The top layer which is solid has to be detached from the tube´s wall by a cotton wool stick. The processing tube II is carefully decanted. Let it drain well. Do not shake in order to avoid loss of the parasite concentrate

9. Resuspend the parasite concentrate according to demand with 0,05-0,5 ml physiological saline solution or Medium A (AF/SAF/ECO/Bailenger) by repeated aspiration, using the one-way pipette. If the concentrate sediment is sticky, it should be diluted using more solution in order to obtain enough transparency for the microscopical examination. For more intensive staining of the concentrated parasites please use high diluted (1:10) Medium C.

10. For diagnosis usually 1-2 drops of concentrated stool suspension dilution per slide will be sufficient. We recommend reading minimum 2 slides for an optimal diagnostics. The cover-glass preparation is now ready for the microscopic diagnostics. Make sure, that your preparation is not too thick

Quality control

For later quality control or storage of the concentrated parasites please use the additional screw cap for processing tube II. Please screw the cap firmly to avoid desiccation.

C. COCCIDIAN STAIN

Principle: method of staining stool smears to screen for intestinal coccidian parasite infections. The coccidian bodies emit a bright yellow fluorescence when activated with a short wave ultraviolet light. The potassium permanganate counter stain causes nonspecific background debris to fluoresce a pale yellow, in contrast with the bright yellow appearance of the coccidian bodies.

Method

1. Arrange slides in serial order, smear side up
2. Keep a finger-thickness between smears
3. Flood with filtered 0.1% Auramine solution and do not Heat
4. Leave for 20 minutes
5. Gently rinse with water, drain
6. Apply decolorizing solution 0.5% Acid alcohol, for 2 min.
7. Rinse, drain
8. Apply 0.5% potassium permanganate solution for 3 min.
9. Rinse, drain

10. Air dry
11. Scan the stained smear systematically at least one length(aprox.30 microscope fields) has to be scanned before reporting a negative, corresponding to 30 high-power fields and taking 1-2 minutes (20x – 40x objective).The positive result should be reported by grading (20X or 40X).Subdued(depressed) lighting is preferable for reading slides.

Interpretations of the result

If fluorescing, structurally identical to coccidian parasite (bright yellow appearance) is seen after reading recommended fields of microscope, positive result with grade will be reported. If not that means pale yellow background with absence of bright yellow appearance) is Negative.Use both positive (+1) and Negative samples at the same time. Also do IQC Whenever you receive new batch of reagent and prepare working reagent. Check for the number and color of intestinal coccidia parasite.

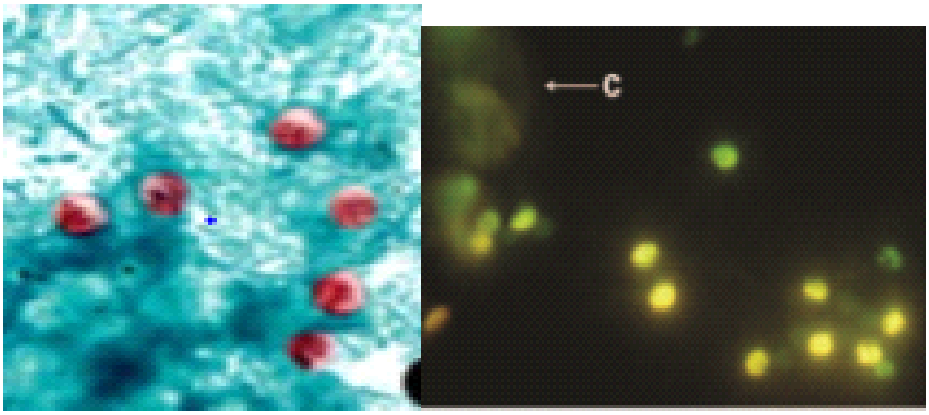
D. Modified Z-N staining method

Principle: This technique is useful for the identification of oocysts of the coccidian species (Cryptosporidium, Cystoisospora, and Cyclospora), which may be difficult to detect with routine stains such as trichrome. Unlike the routine Modified Z-N, this stain does not require the heating of reagents for staining.

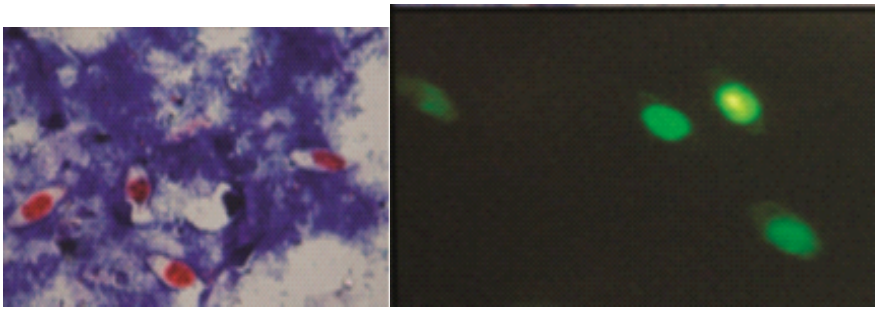
Procedure

1. Prepare a smear from the sediment obtained by the ParasiTrap®oocyst concentration technique
2. Air dry it, and fix the smear with methanol for 1 minute and allow to dry
3. Stain with unheated carbol fuschin for 30 minutes and wash off the stain with water
4. Decolorize with 1% acid alcohol for 1-2 minutes and wash off with water .
5. Counter stain with 0.3% malachite green (methylene blue) for 60 seconds and wash off with water.

Annex VI: Photomicrographs of Cryptosporidium species (A) and I. belli (B) at 200 magnification for fluorescent microscope (Auramine O) and 1000 magnifications by light microscope (modified Z-N).

- 

Modified Zeihl-Nelsen Auramine O

- 

Modified ZN Auramine O

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Annex VII: Declaration

Title of project: Intestinal coccidian parasite prevalence and associated risk factors among diarrhogenic HIV infected patients on ART in Asella and Adama Teaching Hospitals, Ethiopia.

I, the undersigned student of medical Parasitology, declare that this research thesis is our original work in partial fulfillment of the requirement for the master science in Medical Parasitology.

Principal investigator: Dagaga Kenea

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Email: Dagagakenea@gmail.com

This thesis has been submitted with my approval as a **University advisor**;

Date of Submission: _____

Name of primary advisor: Dr Tadesse kebede (PhD, Assistant professor)

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