DIFFERENTIATION OF *Entamoeba histolytica* AND *Entamoeba dispar*
COMPLEX BY MULTIPLEX POLYMERASE CHAIN REACTION

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A Thesis Submitted to the Graduate School in Partial Fulfilment for the
Requirements of the Award of Master of Science Degree in Medical Parasitology of Egerton
University

EGERTON UNIVERSITY

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DECLARATION AND RECOMMENDATION

DECLARATION

This MSc thesis is my original work and has not been submitted or presented to any other university for an award of a degree.

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SM17/2841/2010

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RECOMMENDATION

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DEDICATION

To my beloved wife of Jane and my children Caroline, Catherine and Chris who have patiently moved with me through the journey of making this dream a reality.

ACKNOWLEDGEMENTS

ABSTRACT

Entamoeba histolytica is the causative agent of human amoebiasis. It is one of the most common parasitic infections worldwide, third only to malaria and schistosomiasis. Amoebiasis infects approximately 50 million people causing 40,000 to100,000 deaths per annum. In Africa, infection varies in different countries depending on their social economic factors. Clinical manifestation of amoebiasis is due to existence of two morphologically identical species but have different biochemical and genetic makeup. *Entamoeba dispar* is a commensal species, while *Entamoeba histolytica* is a pathogenic one, but both of them can occur together during incidences of infection. Thus, it is of clinical importance to differentiate between the two species, for correct treatment decision, management and public health. Infection occurs mainly by ingestion of viable cysts from contaminated sources such as water and food. The main objective of this study was to differentiate *Entamoeba histolytica* and *Entamoeba dispar* by multiplex polymerase reaction in stool samples. A descriptive survey research was adopted between January 2012 and April 2012 and one hundred and sixty nine (169) freshly collected stool samples from patients seeking medical services at outpatient department in Naivasha District hospital with symptoms of amoebiasis were analyzed using microscopy and multiplex polymerase chain reaction techniques. Data for the presence of the two species of *Entamoeba* were analysed using multivariate statistics and Chi-square ($\chi2$). Microscopy detected 36 (21.3%) patients were infected with *E. histolytica /Entamoeba dispar* complex cysts or trophozoites. On the other hand, multiplex polymerase chain reaction identified 42 (24.9%) patients who had DNA of *E. histolytica* or *Entamoeba dispar* in their stool samples. Mono infection with *Entamoeba dispar* was the highest with 34 (20.1%), followed by *Entamoeba histolytica* 4 (2.4%) and co-infection with both species at 4 (2.4%). Generally multiplex polymerase chain reaction technique reduced the chances of misdiagnosis by 9 (6.7%) patients. The study showed that multiplex polymerase chain reaction is a useful diagnostic tool for distinction between *Entamoeba histolytica* and *Entamoeba dispar* complex as well as the presence of mixed infection simultaneously in a single polymerase chain reaction steps in laboratory analyses. Despite the cost of carrying out the technique being higher compared to conventional microscopy, multiplex polymerase chain reaction is more sensitive and specific in detection and differentiation of *Entamoeba histolytica* and *Entamoeba dispar*. To the best of my knowledge this is the first report to differentiate *Entamoeba histolytica* and *Entamoeba dispar* from human faecal samples from Kenya and its implications are further discussed.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ALA   Amoebic liver abscess

bp   Base pair

EDP1   *Entamoeba dispar* Primer 1

EDP2   *Entamoeba dispar*   Primer 2

EHP1   *Entamoeba histolytica* Primer 1

EPH2   *Entamoeba histolytica* Primer 2

Gal/GalNAc   Galactose / N-acetyl-galactosamine

MoH   Ministry of Health

MPCR   Multiplex Polymerase Chain Reaction

MUC2   Mucin 2

WHO   World Health Organisation

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Entamoeba histolytica belongs to the family *Entamoebidae* and is the primary causative agent of human amoebiasis. It is one of the most common parasitic infections worldwide, third only to malaria and schistosomiasis (Kurt *et al.,* 2008). *Entamoeba histolytica* infects approximately 50 million people worldwide causing 40,000 to 100,000 deaths per annum (Calderaro *et al.,* 2006). There are cases where up to 50% of the population may be affected in regions with poor sanitary conditions (Saeed and Manal, 2007). It has been estimated that amoebiasis affects about 10% of the global population of which, 90% will show no clinical symptoms (Gonin and Trudel, 2003). These figures appear to vary from country to country and in different regions. For example, studies undertaken in different countries showed variable infection rates as follows: in Pakistan 21.69% (Tasawar *et al.,* 2010), Egypt >21% (Stauffer *et al.,* 2006), Côte d'Ivoire 18% (Quattara *et al.,* 2010), Nigeria 14.3%, (Dawah *et al.,* 2010), Brazil 21% (Santos *et al.,* 2007), Kenya 12.6% (Nguhiu *et al.,* 2009). All these studies report different infection rates depending on the procedure that was used therein.

Clinical manifestations of amoebiasis infections are due to the existence of two morphologically identical species of *Entamoeba*, but with different biochemical and genetic makeup. The two species of *Entamoeba* include the non pathogenic *Entamoeba dispar* and pathogenic *Entamoeba histolytica* (Fotedar *et al.,* 2007a). It has been stated in other studies that the infection by *E. histolytica* may either be asymptomatic or symptomatic resulting in dysentery or in extreme cases extra intestinal diseases involving other organs such as the liver, lungs or brain (Salles *et al.,* 2003; Fotedar *et al.,* 2007a). The diagnosis of *E. histolytica* infection in the laboratory has traditionally relied on microscopic examination of fresh or fixed stool specimens (Stark *et al.,* 2008). However, microscopy has several limitations, for instance its inability to distinguish the pathogenic species from non pathogenic ones and high chances of false-positive results due to misidentification of macrophages as trophozoites or polymorphic nuclear leukocytes as cysts and other *Entamoeba* species (Rashed *et al.,* 2011).

The concerns associated with the limitation of microscopy, necessitated the search for more specific and sensitive alternative methods that are diagnostic nucleotides such as polymerase chain reaction (PCR). These are in addition to enzyme based cultures and antigen- antibody based enzyme linked immunosorbent assays (ELISA). All these have been

introduced for the purpose of diagnosis and separations of species within *Entamoeba* complex (Ali *et al.,* 2012). These new techniques have been employed with some level of shortcoming which leads to varied diagnostic outcomes. For example, cultures with isoenzymes are not widely available and are impractical for routine diagnostic laboratories because it takes too long to detect the presence of the parasite (Kheirandish *et al.,* 2011); time which may not be practical especially for patients who often show symptoms and may often require immediate intervention. Therefore, correct diagnosis is vital not only in reducing human mortality and morbidity but more importantly to avoid unnecessary treatment of patients infected with non pathogenic form *E. dispar* (Rashed *et al.,* 2011). The current circumstances present a challenge as the statistics of global prevalence of *E. histolytica* (amoebiasis) may not be absolutely reliable since much of this information was generated in the era when microscopy was the only method for diagnosis, given its technical limitation in separating members within the *Entamoeba* species complex.

The current study, investigated the presence and relative proportions of the two members of *Entamoeba* complexes in clinical fecal specimens (from patients who presented with gastrointestinal symptoms) by microscopy and afterwards by multiplex polymerase chain reaction in order to compare the sensitivity of the two techniques in detection and identification of the predominant species (pathogenic *E. histolytica* and non pathogenic *E. dispar)* of the complex.

1.2 Statement of the Problem

Amoebic dysentery is one of the most debilitating disease worldwide only third to malaria and schistosomiasis. Its health burden is exacerbated by the fact that the causative agent is often confused with its close relative *E. dispar*; a morphologically similar but harmless commensal in the human gut. The main issue with misdiagnosis can often lead to misuse/abuse of drugs often caused by administration of drugs to patients who may be having diseases other than amoebiasis, or in other cases may only be having the non pathogenic form. This study explored the use of multiplex PCR, a more robust and sensitive technique which appears promising in its ability to separate the two species. This is particularly important considering that in nearly all hospitals in Kenya; microscopy remains the method of choice for diagnosis.

1.3 Objectives

1.3.1 General objective

To differentiate between *E. histolytica* and *E. dispar* complex in stool specimens using multiplex polymerase chain reaction.

1.3.2 Specific objectives

1. To distinguish between the pathogenic *E. histolytica* from non pathogenic *E. dispar* by multiplex polymerase chain reaction.
2. To determine the incidence of *E. histolytica* and *E. dispar* in Naivasha Sub County.
3. To compare the sensitivity between multiplex polymerase chain reaction and microscopy methods in techniques of *E. histolytica and E. dispar* complex.

1.4 Hypotheses

1. There is no genetic difference between pathogenic *E. histolytica* and non pathogenic *E. dispar* using multiplex polymerase chain reaction technique.
2. There is no difference in the incidences of *E. histolytica* and *E. dispar* in Naivasha Sub County.
3. There is no difference in sensitivity between multiplex Polymerase chain reaction and microscopy in diagnosis of *E. histolytica* and *E. dispar* complex.

1.5 Justification

Entamoeba species complex comprising among others species *Entamoeba histolytica* and *Entamoeba dispar* are morphologically identical organisms but only differ biochemically and genetically. *Entamoeba histolytica* is recognized as a pathogen while the status of *Entamoeba dispar* remains unclear hence it is primarily considered a commensal. In Kenya, microscopy remains the technique of choice for diagnosis of species within the *Entamoeba* complex. In light of our present knowledge, microscopy must be considered as a screening method for the *E. histolytica* and *E. dispar* complex and not as a technique to confirm their diagnosis. However, majority of laboratories in health facilities lack the capacity to differentiate between *E. dispar* and *E. histolytica* and often report the two species complex as *E. histolytica*. Based on these facts the prevalence of *E. histolytica* and *E. dispar* is questionable, because microscopically the two species are indistinguishable. Consequently, it was important that this study was undertaken to differentiate *E. histolytica* and *E. dispar* complex and determine their proper/actual incidences. In addition, the sensitivity and specificity of the two techniques for accurate diagnosis was determined.

1.6 Expected Outputs

1. The pathogenic and non pathogenic *Entamoeba* species distinguished genetically and their incidence in Naivasha Sub County established.

2. Publication in peer-reviewed journals.

3. Award of Master of Science degree in Medical Parasitology

CHAPTER TWO

LITERATURE REVIEW

2.1 Amoebiasis

Entamoeba histolytica is an entero-parasite and the primary cause of a disease called amoebiasis in human, which is largely endemic in developing countries (Lejeune *et al.,* 2009). The first case of dysenteric disease in man, caused by amoeba was described in 1875 by the Russian physician Friedrich Losch. He described the motility of the amoeba and the typical nucleus and ingested red blood cells reassures us that he was actually looking for the trophozoite of what now is recognised as *E. histolytica*. He first named it *Amoeba coli* because it appeared in the colon. In 1903, Fritz Schaudinn changed the name to *E. histolytica* due its ability of the amoeba to cause tissue lysis (Pinilla *et al.,* 2008). The distinction between *E. histolytica* and *E. dispar* was first suspected by Brumpt in 1925 who then suggested that the differences in symptoms and global distribution of invasive amoebiasis were due to the presence of two morphologically identical species of amoebae, the pathogenic and non-pathogenic forms (Kurt *et al.,* 2008). The infection of *E. histolytica* affects about 10% of the global population of which 90% are asymptomatic (Gonin and Trudel, 2003).

In a study carried out in Kibwezi Sub County in Makueni County in Kenya, *E. histolytica* and *E. dispar* complex infection is estimated to affect about 13% of population (Nguhiu *et al.,* 2009). However, the prevalence of the *Entamoeba* complex in these subs Counties and generally in Kenya is unknown. In other countries, the prevalence of *Entamoeba* complex is known. In a study undertaken on stool samples from 49 patients who had been diagnosed with amoebiasis in Cuba; multiplex polymerase chain reaction showed 75.5% of the diagnostic fragments were characteristic of *E. dispar* (96bp) while the remaining 24.5% showed both *E. histolytica* (132bp) and *E. dispar* (Nunez *et al.,*2001). While in Brazil, eleven stool samples out of twenty seven were identified positively by multiplex-PCR out of which nine (81.8%) presented the diagnostic fragment characteristic of *E. dispar* (96 bp) and two (18.8%) had *E. histolytica* (132 bp). The remaining sixteen samples (59.2%) had unknown DNAs. In addition, the samples were further examined microscopically and among the negative samples detected by microscopic examination, three were positive for *E. dispar* and one positive for *E. histolytica* (Santos *et al.,* 2007). This is likely to imply that: multiplex is superior and specific in detection and differentiation of the two species. On the other hand, in Iraq out of one hundred stool samples processed 43% had

diagnostic fragment characteristic of *E. dispar* (96bp) while 26% had diagnostic fragment characteristic of *E. histolytica* (132bp). The remaining 24 stool samples which were found negative following microscopic examination were subjected to multiplex PCR. One stool sample was found to have diagnostic fragment characteristic of *E. histolytica* while another four had the diagnostic fragment characteristic of *E. dispar*, the remaining 19 were confirmed negative (Aseel and Sarmad, 2010). In Egypt, multiplex PCR detected (36%) while microscopy was (25%) in hundred stools samples analysed. Multiplex PCR identified (25%) with diagnostic fragment were characteristic of *E. histolytica* while (41.7%) had the fragment characteristic of *E. dispar* (96bp) and another (33.3%) had both *E. dispar* and *E. histolytica* (Mona *et al.,* 2011).

2.1.1 Mode of infection

There are several possible avenues of infection by *Entamoeba* species but the main route is the fecal-oral route where edible materials contaminated with viable cysts are ingested (Singh *et al.,* 2009). For instance, direct contact with dirty hands, poor food handling, use of untreated human faeces as fertilizers such as sewerage water for growing vegetables which harbour the viable cysts (Ejaz *et al.,* 2011). It has also been reported that infection may also occur via oral-anal sexual practices or event direct rectal inoculation through colonic irrigation devices (Sirikelum and Kodikara, 2011). Recently, Biswapriya *et al.,* (2011), presented evidence that infective cysts may be spread by arthropods such as cockroaches and flies, thus suggesting that these insects are able to play a rare but important role in physical transmission

2.1.2 Life cycle of *Entamoeba histolytica*

Entamoeba histolytica has a simple life cycle (Figure 2.1) that comprises of an infectious cyst form, an amoeboid trophozoite stage and a mature cyst (the infective stage) (Figure 2.2). It is the mature cysts (the infective stage) which are ingested in fecal contaminated material: food, water (Ejaz *et al.,* 2011). Once ingested, encystation takes place in the small intestines after which the trophozoites (Figure 2.3) are released and they migrate into the large intestines. Once in the large intestines, the trophozoites multiply by binary fission producing numerous cysts which are passed out in the faeces. The cysts have a thick wall made partly of chitin which enables them to survive for days to weeks in the external environment (Varki *et al.,* 2009). In cases where the human patient has diarrhoea, the trophozoites are passed out in stool but are rapidly destroyed once outside the body. In such

cases even if they are ingested by the next host, they do not survive the gastric environment as they are confined in the intestinal lumen and do not cause any symptoms. A person is termed as having amoebiasis only when the trophozoites disrupt the mucosal barrier and penetrate the colon space causing ulceration. In other cases, the trophozoites may invade the intestinal mucosa and other organs such as liver, brain and lungs (Ravdin and Stauffer, 2006).
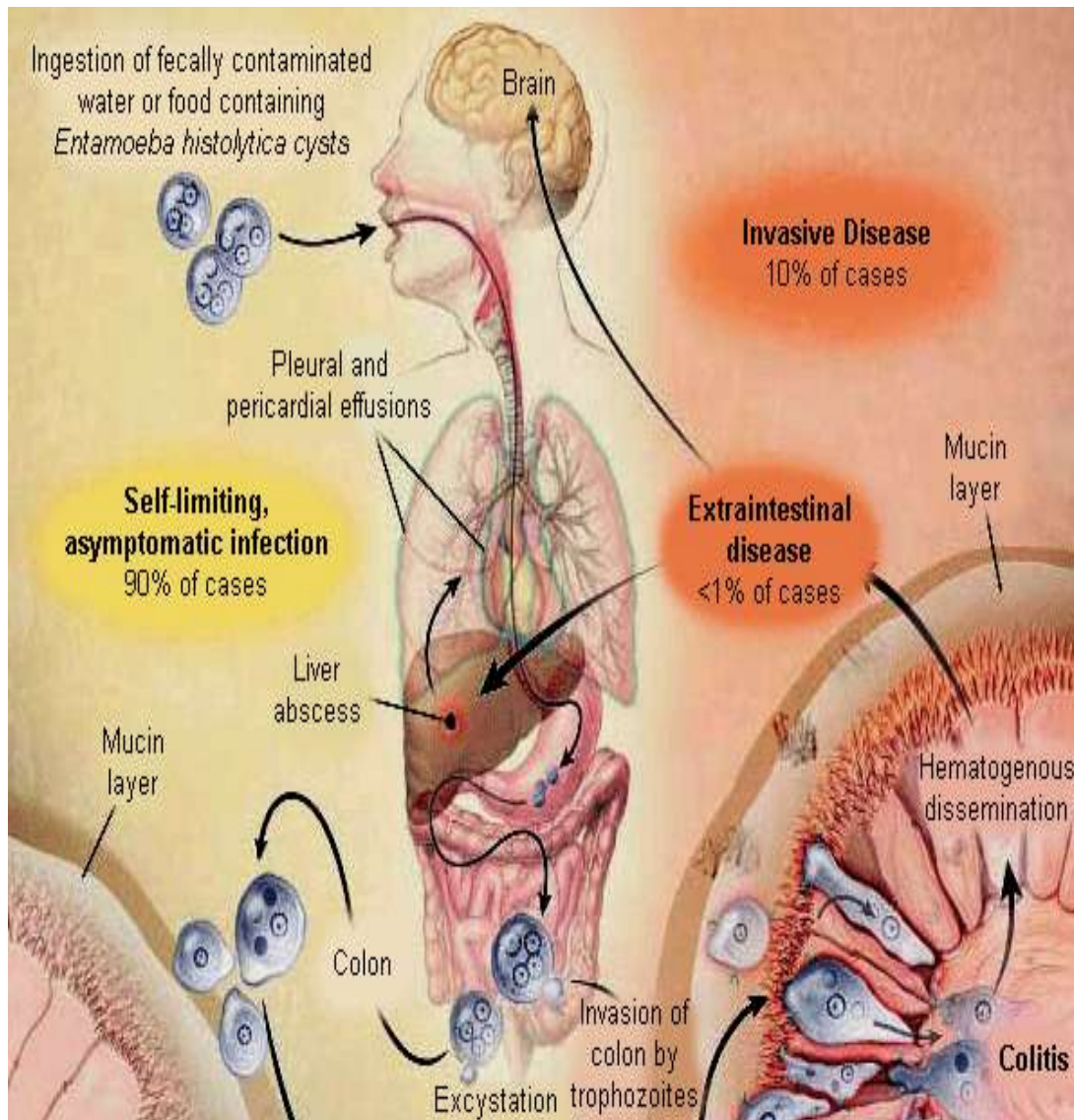

Figure 2.1: Life cycle of *E. histolytica* (Rashidul *et al.,* 2003).
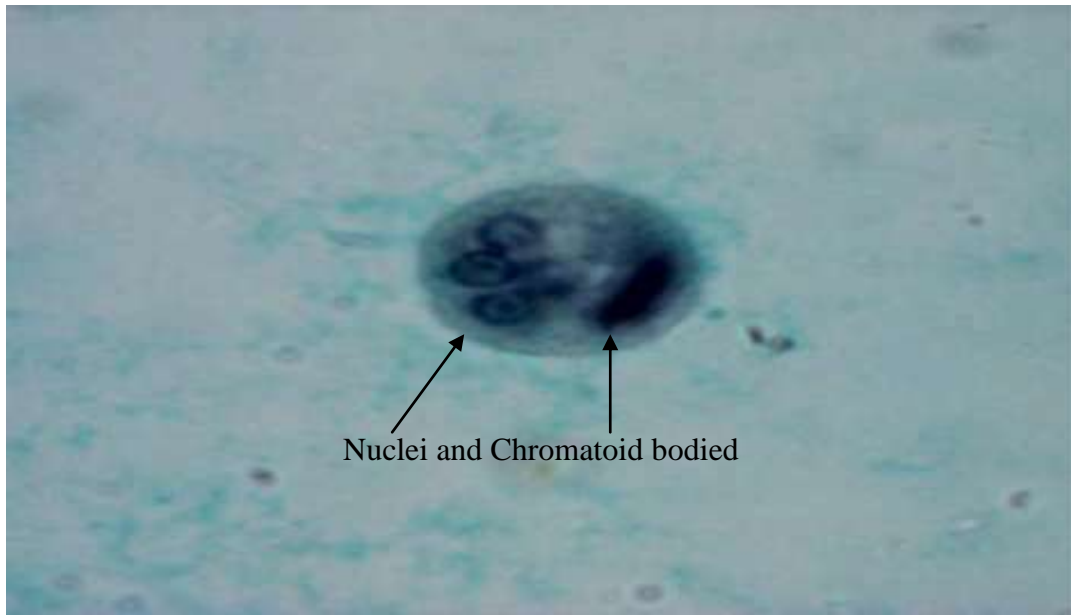
Figure 2.2: Stained trichrome cysts of *E. histolytica* with three visible nuclei and a
Chromatoid body (Lebbad *et al.,* 2005)

Figure 2.3: Trophozoites of *E. histolytica* with ingested erythrocytes**.** ((Lebbad *et al.,* 2005)

2.1.3 Pathology of *Entamoeba histolytica*

Entamoeba histolytica trophozoite frequently lives within the large intestine with no clinical overt symptoms. Infections of *E. histolytica* vary in intensity from asymptomatic to severe or fatal invasions. The non-invasive trophozoites are often asymptomatic infections and responsible for the spread of the parasite with numerous cysts being passed in normal stools, diarrheic stools primarily contains trophozoites. Some of trophozoites invade the colonic epithelium, leading to the formation of amoebic ulcers, while others invade the intestinal mucosa affecting other organs thereby resulting into complications in the affected organs and death (Levecke *et al.,* 2010). The development of symptomatic disease results from mucosal necrosis caused by liberated lysosomes enzymes. This results in the escape of red blood cells which are ingested by the trophozoites. These results in severe diarrhea with blood and mucus present and the patient will present symptoms such as abdominal pain or cramps, tenesmus fever and vomiting (Dooron *et al.,* 2005). The non-invasive infection can persist or progress to an invasive disease in which trophozoites penetrate the intestinal mucosa and kill the epithelial cells. The galactose and N-acetyl-D-galactosamine (Gal/GalNAc)-specific lectin moieties mediate attachment to epithelial cells and mucin. After substrate adherence, amoeba rapidly induces apoptosis and cytolysis of host cells (Sateriale and Huston, 2011). Some of the outcomes of these penetrations include local abscesses and peritonitis, secondary infections with bacteria and formation of ameboma. The lesions observed during amoebiasis are caused by harmful products such as protein and or oligosaccharide components of the mucin-2 (MUC2) polymer secreted by trophozoites and possibly by host defence (Cassia *et al.,* 2010). Also some trophozoites progress to a systemic or extra intestinal infection via the blood stream (Stanley, 2003). These trophozoites affect liver, lungs and brains.

2.2 Laboratory Diagnosis of *Entamoeba histolytica*

In past decades, microscopy has remained the preferred method of choice for diagnosing intestinal *E. histolytica* infection. Despite its limitation in differentiating between members of the *Entamoeba* complex, it has remained the technique of choice in many parasitological laboratories worldwide (Fotedar *et al.,* 2007a). In light of our present knowledge, microscopy should be considered as a screening method for the *E. histolytica* and *E. dispar* complex but not as a technique to confirm the diagnosis of *E. histolytica* (Santos *et al.,* 2007). Its sensitivity is about 60% compared to molecular, culture and immunological techniques (Ali *et al.,* 2008). The common concentration technique used in Kenyan

laboratories is formal-ether technique that was developed several decades ago by Ritchie, 1948. Although, slight modifications have been made since that time the principle remains unchanged except that ether has been replaced by ethyl acetate (Young *et al.,* 1979) and later on sodium acetate-acetic acid-formalin (SAF) may be used instead of formalin as the fixative (Ghazanchaei *et al.,* 2012).

It is estimated that less than 1% of *E. histolytica* trophozoites invade the intestinal mucosa affecting other organs leading to extra intestinal amoebiasis that results in complications in the affected organs and death (Levecke *et al.,* 2010). There exists a challenge for diagnosis of patients with extra-intestinal amoebiasis using stool specimens because they rarely have *E. histolytica* parasites in their stool samples (Fotedar *et al.,* 2007b). For this reason, more appropriate and sensitive techniques have been developed and include molecular and antibody detection techniques, (e.g. enzyme-linked Immunosorbent assay). Recently, in a study conducted in Bangladesh, it was indicated that a sensitivity of 96% for amoebic liver abscess patients was achieved but with only 46% for amoebic colitis patients (Haque *et al.,* 2010). These means the antibody-antigen techniques are best techniques for detecting extra-intestinal amoebiasis. Microscopy offers very low sensitivity (60%) whereas molecular techniques have higher sensitivity and specificity of 94% and 100% respectively.

2.3 Techniques for differentiation of *Entamoeba* species

2.3.1 Zymodemes

This is a technique used to differentiate *E. histolytica* and *E. dispar* in stool or liver abscess specimens. It was the first procedure to be used to characterize different *E. histolytica* isolates. However, it did play a major role in the early differentiation of *E. histolytica* and *E. dispar* (Sergeant *et al.,* 1978). It involves culturing stool samples, rectal biopsy specimens or liver abscess in axenic culture media such as TY1-S-33 in the absence of any other metabolizing cells (Clark *et al.,* 2002). If amoebic trophozoites are present, they would be visualised on the wall of the test tube or in the debris. The indentified trophozoites are differentiated using specific isoenzymes such as hexokinases by electrophoresis. The main drawback of this procedure is that it is tedious and time consuming (Intarapuk *et al.,* 2009). Furthermore, the sensitivity and accuracy is low because many samples that are positive by microscopy are culture negative (Fotedar *et al.,* 2007a).

2.3.2 Antigen-Antibody detection

These are commercially available antigen detection kits such as *Entamoeba* CELISA PATH and TechLab *E. histolytica* II both of which use monoclonal antibodies against Gal/GalNAc-specific lectin an adhesin molecule of *E. histolytica* (Fotedar *et al.,* 2007a). Studies carried out in Bangladesh, an area with high *E. histolytica* transmission have shown that antigen detection has a high sensitivity equal to PCR (Haque *et al.,* 1998). However, in investigations carried out in non-endemic areas demonstrated a poor sensitivity for antigen detection compared to PCR (Stark *et al.,* 2008). This is an indication that antigen testing has the benefit of being simple, rapid and yielding good results in areas with high prevalence but it is limited in settings where there are few cases of *E. histolytica* infections (Visser *et al.,* 2006; Stark *et al.,* 2008).

2.3.3 Molecular Approaches

These involve modern, powerful, highly sensitive and useful methods not only for differentiation of *E. histolytica* and *E. dispar* but for genetic typing of isolates as well. There are several PCR–based methods have that been developed and tested in diagnostics. However, they are time-consuming, expensive and require specialized skills and practical experience (Haque *et al.,* 1998). PCR is used to amplify a specific region of DNA strand (the DNA target). The amplification of the fragments varies with different isolates (Muller *et al.,* 1997). DNA extraction performed on cultured trophozoites has minor challenges compared to the DNA extracted directly from stool samples. This is because feces contain several PCR inhibitors (Abu and Radstrom, 2000) most of which require additional step of inhibitor removal by an optimal extraction procedure. However, with current development and advances in molecular biology, this step that requires inhibitors removal can be overcome by using more sensitive commercial kits. Commercial spin columns (QIAampTM DNA mini kit or QIAampTM DNA stool mini kit) are among the most widely used devices for extraction of *Entamoeba* DNA directly from stool samples (Gonin and Trudel, 2003). The extraction procedures involve cyst disrupting steps such as bead-beater, thawing, boiling, and freezing treatment. However, manual extraction is time consuming and inconvenient when analyzing a large number of samples. That notwithstanding, molecular biology techniques are now becoming part of routine diagnostic procedures for detecting intestinal parasites. These current techniques are amenable to automation especially as pertains to the DNA extraction procedures (Calderaro *et al.,* 2010).

Some of the earliest methods for differentiating *E. histolytica* from *E. dispar* included two single PCRs that targeted either the small subunit ribosomal RNA (ssrRNA) gene (Clark and Diamond, 1991) or the gene encoding peroxiredoxin (a 30-kDa protein) (Tachibana *et al.,*1991). The single two single PCR techniques remain the most commonly used worldwide (Fotedar *et al.,* 2007a). Other more current protocols are available for detection and differentiation of *E. histolytica* and *E. dispar*, which include duplex PCR, multiplex PCR with sensitivity and specificity of 94% and 100% respectively (Khairnar and Parija,2007; Nazemalhosseini *et al.,* 2010). Nested PCR and real-time PCR are more sensitive, more rapid than conventional PCR leading to shorter turnaround times with much reduced risk of amplicon from laboratory environments. More importantly they have significant reduced reagent costs (Qvarnstrom *et al.,* 2005). The Principle of multiplex PCR is that more than one target sequence can be amplified by including more than one pair of primers in the reaction. It saves time and effort within the laboratory setup and without compromising test quality (Elnifro *et al.,* 2000). The conventional PCR basically tells whether or not a gene of interest is in the sample. This is done semi-quantitavely if the PCR is done in a low number of cycles it will indicate whether one sample expresses more of the gene of interest than another sample. The amplified products can be visualized afterwards by agarose gel/ethedium bromide electrophoresis.

2.4 Amoebiasis Management

There are several management options for amoebiasis including: pharmacological, surgical intervention and preventive measures. According to the WHO recommendation the administration of anti-amoeba drugs should be done only after differentiation of *E. histolytica* and *E. dispar* WHO, (1997). It is further recommended that whenever possible no patient should be treated on the basis of microscopic findings alone (Santos *et al.,* 2013). In addition, all cases identified as *E. histolytica* regardless of symptoms should be treated due to the risk of the invasive disease further spreading. On the other hand, cases found involving only *E. dispar* should not be treated (Stanley, 2003). If a patient with *E. dispar* has intestinal symptoms other investigations should be carried out to find other possible causes of the disease. Asymptomatic *E. histolytica* infection should be treated with a luminal amoebicide such as diloxanide furoate or paromomycin while invasive intestinal or extra-intestinal amoebiasis should be handled by administering a tissue amoebicide such as metronidazole followed by luminal treatment (Blessmann *et al.,* 2006). Surgical and percutaneous

intervention is required in cases of acute abdominal disturbances such as perforated amoebic colitis, massive gastrointestinal bleeding and toxic megacolon (Gutierrez *et al.,* 2010).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Research Design

A survey was carried out in order to establish the correlations between age, gender, locations, positive and negatives. Brief interviews were used in collecting data since they are useful in investigating issues in an in depth way and also because they usually achieve a high response rate.

3.2 Study Area

Samples were obtained between January 2012 and April 2012 at Naivasha District hospital in Nakuru County about 90 km from Nairobi, the capital city of Kenya. Naivasha is one of the sub counties in Nakuru County (Figure 3.1). It covers an area of 1707 Km^2 with a population density of approximately 350,000 people according to 2009 population census (KNBS, 2009). It lies along the Trans Africa's Great North Road which runs from Mombasa through Nairobi, Uganda, Rwanda and Zaire. It is located on the shore of Lake Naivasha (0° 43' 0" South, 36° 26' 0" East).

Figure 3.1: A map showing the location of Naivasha District hospital in Naivasha sub County, Nakuru County (Source: maps google.co.ke, 2011).

3.3 Sampling procedure

In the current study, selection criteria focused on patients who had symptoms of colitis, bloody or mucous diarrhoea or amoebic dysentery fever at the District hospital as participants for the study. Sample size was determined following the method proposed by Cochran, (1977). The number of patients to be sampled for the study was derived from the formula shown below:

$$n = \frac{Z^2 pq}{d^2} \quad \text{(Cochran, 1977)}$$

Where n was the desired sample size, Z was the standard normal deviation at the required confidence that have the characteristic of being measured, p was the target population estimated to have characteristics being measured, d was the level of statistical significance set, q was 1-p.

Where;

n = unknown

Z = 1.96

p = 12.6 %(0.126)

d = 0.05

q = 1-p (1-0.126) = 0.874

$$n = \frac{1.96 \times 1.96 \times 0.126 \times 0.874}{0.05 \times 0.05} = \frac{0.4231}{0.0025} = 169$$

n = 169

Using the above formula, 169 patients were included in the study. Sampling was done from patients seeking medical services at outpatient department in Naivasha District hospital. After obtaining informed written consent from the patients, specimens were collected from symptomatic patients presenting any of the following: colitis, bloody or mucoid diarrheal or amoebic dysentery fever. Only male and female patients aged between 2 to 60 years who had not used any anti-amoeba drugs within one week (prior to observation in the hospital) were included in the study. Adults gave informed consent while for children between 2 and 18 years, their parents or guardians gave consent and signed. Clean dry faecal containers were given to them or their parents or guardians and instructions given on how to collect the stool specimen. Data concerning their gender and ages and location where the patients originally came from were recorded on submission of specimen.

3.4 Sample Analysis

3.4.1 Microscopy

Two grams of each sample were immediately aliquoted into 1.5ml screw-cap tube and stored in a freezer at -20°C. The samples were placed in cold chain boxes and transported to U.S Army Medical Research Unit Microbiology-Hub Kenya-Kericho for PCR analysis. Another two grams of the faecal samples were used for parasitological examinations at the hospital diagnostic laboratory in Naivasha District hospital. Direct wet smear and formal-ether concentration techniques were performed within two hours after collection. From each sample, two wet smear and one from formal-ether concentration were prepared according to the protocol previously described by the WHO (2006). The smears were then examined under a light microscope for identification at 10X then for confirmation at 40X. Results were recorded either as positive, if cysts or trophozoites of either species (*E. histolytica/E. dispar*) were detected or negative if none were detected.

3.4.2 Multiplex-Polymerase Chain Reaction

DNA Extraction

DNA was extracted from 150 mgs of stool samples using ZR Faecal DNA MiniPrep™Catalogue No. D6010 kit, according to the manufacturer's Protocol (ZYMO RESEARCH CORP). Briefly, Zymo-spin™ IV-HRC Spin filter (green top) were prepared prior to use by snapping off the base and inserted into a collection tube and centrifuged in a micro centrifuge at 8,000 xg for 3 minutes. The faecal material was placed in a ZR Bashing Bead™ Lysis and 750 µl lysis solutions added to the tube. The mixture was then vortexed at high speed for 5 minutes. The ZR Bashing Bead™ was then centrifuged at 10,000 xg for 1 minute in a micro-centrifuge, 400 µl of collected supernatant was transferred into a Zymo-spin™ IV spin filter (orange top) in a new collection tube and centrifuged in a micro-centrifuge at 7,000 xg for 1 minute. Twelve hundred microliters of faecal DNA binding buffer was added to the collected filtrate in the collection tube mixed and 800 µl of the mixture was transferred into a new Zymo-spin™ IIC column in a new collection tube and centrifuged in a micro-centrifuge at 10,000 xg for 1 minute. The filtrate in the collection tube was discarded and the remaining 400 µl of the mixture was transferred to the same Zymo-spin™ IIC column in a collection tube and centrifuged at 10,000 xg for 1 minute. Zymo-spin™ IIC column was placed in a new collecting tube and 200 µl DNA Pre–wash buffer added and centrifuged in a micro-centrifuge at 10,000 xg for 1 minute. Five hundred microliters of faecal DNA wash buffer was added into Zymo-spin™ IIC column and centrifuged at 10,000

xg for 1 minute. Zymo-spin™ IIC column was transferred into a clean micro-centrifuge tube and 100 µl DNA elution buffer added directly to the column and centrifuged 10,000 xg for 30 seconds to elute the DNA. The eluted DNA was transferred into minute Zymo-spin™ IV-HRC Spin filter in a clean 1.5 ml micro-centrifuge tube and centrifuged at 8,000 xg for 1 minute. The filtered DNA was stored at -20°C before PCR analysis.

3.4.3 Estimation of DNA Integrity

The purity of DNA was assessed using NanoDrop 2000 spectrophotometer (Thermo Scientific). Then, 2 µl of the extracted DNA was loaded into the lower measurement pedestal of the NanoDrop spectrophotometer and optical density measured. All the samples were within the ratio 260/280 nm optical density (1.8-2.0). The concentration of the DNA was also estimated using 1.0% w/v agarose gel electrophoresis (Sigma, UK). One gram of agarose powder was weighed and put in a flask and 100ml of 1X Tris-Borate-EDTA (89Mm Tris-HCL (pH 8.3), 89mM Boric acid and 2.5Mm EDTA), the mixture was boiled for quick dissolution then allowed to cool to 50°C and stained with 2 µl Ethedium Bromide. The solution was cast in tray with combs to form indentations (wells about 1mm depth) then allowed to cool for 30 minutes. Afterwards, 2 µl of each sample of the extracted DNA were mixed separately with 1µl of loading dye (50% glycerol, 250Mm EDTA (pH 8.0), 0.01 Bromphenol blue) and loaded into the wells and electrophoresed at 80V for 45 minutes. The gel was visualized under an Alpha Imager[R] Hp 3400 and the results printed using Mitsubishi printer (Figure: 4.2), the extracted DNA were stored at -20°C prior to PCR analysis.

3.4.4 Amplification Reaction

The multiplex polymerase chain reaction was carried out according to the protocol described by Nunez *et al.,* (2001) with some modifications. In a 50 µl reaction contained Dream Taq™ Green PCR Master Mix (2X) 25 µl, 21.25 µl nuclease free water; 0.75 µl of 40pmoles of each oligonucleotide primer and 3 µl of DNA template. Amplification was carried out using a GenAmp PCR system 9700 (Applied Biosystems). Initial denaturation at 94°C for 5minutes followed by 35 cycles of 30 seconds at 94°C annealing at 55°C for 30 seconds and primer extension at 72°C for 40 seconds with final extension at 72°C for 7 minutes. The primers pair used in this study are as stated *E. dispar* (EDP1-5′-ATGGTGAGGTTGTAGCAGAGA-3′and EDP2- 5′-CGATATTGACCTAGTACT-3′) and *E. histolytica* (EHP1-5′ CGATTTTCCCAGTAGAAATTA-3′ and EHP2-5′-CAAAATGGTCGTCTAGGC-3′) and were sourced from Bioneer, South Korea. Dream Taq™ Green PCR Master Mix (2X) contained Green buffer, dNTPs and 4mM $MgCl_2$

(Fermentas Life Sciences, USA) was used. Optimization of Multiplex PCR was done using DNA from HMI-IMSS strain as a positive control for *E. histolytica* and SAW 760 strain as positive control for *E. dispar*. Nuclease-free water was used as negative control. To rule out amplification inhibitors DNAs of the negative samples were spiked with DNAs of positive controls and all turned positive.

3.4.5 Detection of PCR -Amplified DNA Products

The PCR products were resolved in 2 % agarose gel and visualized under Alpha Imager[R] Hp 3400. Ten microliters of amplified PCR products of each sample were loaded separately into 1mm depth wells and 5µl of a molecular marker 100bp (Fermentas Life Sciences, USA) was loaded in one of the wells. The loaded gel was placed in an electrophoresis tank and the left to run at 90V for 60 minutes. The gel was visualized under Alpha Imager[R] Hp 3400.

3.5 Data Analysis

Data of the presence of the two species of *Entamoeba* collected using microscopy and PCR was analysed using multivariate statistics, while the efficiency of the two techniques in detecting presence of the two species were compared using Chi-square (χ2). Test for independence using SPSS version 20 to produce mean scores for incidences of *E. histolytica E. dispar*. The positivity and negativity rates for both techniques were reported in terms of frequencies and percentages. Percentage sensitivity and specificity of the two techniques was determined as expressed below: (Hennekens and Buring, 1987)

$$\text{Sensitivity} = \frac{TP}{TP+FN} \text{ X } 100\%$$

$$\text{Specificity} = \frac{TN}{TN+FP} \text{ } X \text{ } 100\%$$

Where;

TP = True Positive

FN =False Negative

TN= True Negative

FP= False Positive

3.6 Logistical, Legal and Ethical Considerations

In order to collect data from the field, a written research permit from the Ministry of Health- Government of Kenya was sought together with prior written informed consent from each subject according to the requirements of Egerton University Research Ethical Committee. The clearance by the Ministry and Egerton University are attached in appendix 2 and 3 respectively. Other logistical concerns, like pre-field work logistics such as pre-testing the instruments, including making an adjustment tour of the study area to strike rapport with authorities and participants were done before commencement of research.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results

4.1.1 Microscopy

One hundred and sixty nine stool samples were analysed by microscopy for the presence of *Entamoeba* life stages. Thirty six (21.3%) samples were detected to have either *E. histolytica/E. dispar* trophozoites or cysts or both and hence considered positive. One hundred and thirty three (78.7%) samples were negative (Figure 4.1).

Figure 4.1: Microscopy positive samples in a wet mount stained with lugoʼs iodine *E. histolytica/E. dispar* trophozoites and *E. histolytica*/*E. dispar* cysts A and B respectively.

4.1.2 Infected Patients

The 36 (21.3%) patient who were infected with *E. histolytica/dispar* complex after diagnosis with microscopy, were given the following medication; adults Tinidazole 2gm once per oral doses daily for three days, followed by Paromomycin 25-35 mg/kg/day per oral in three doses for seven days. For the children the same drugs were used but their doses were determined after measuring their weight. These was done because *E. histolytica/dispar* complex was not differentiated immediately, to avoid the risk of invasion by *E. histolytica* trophozoites. Although, those drugs have minimal toxicity, the patients were adviced to see the clinician in case they had any adverse reaction to the drugs.

4.1.3 DNA Integrity
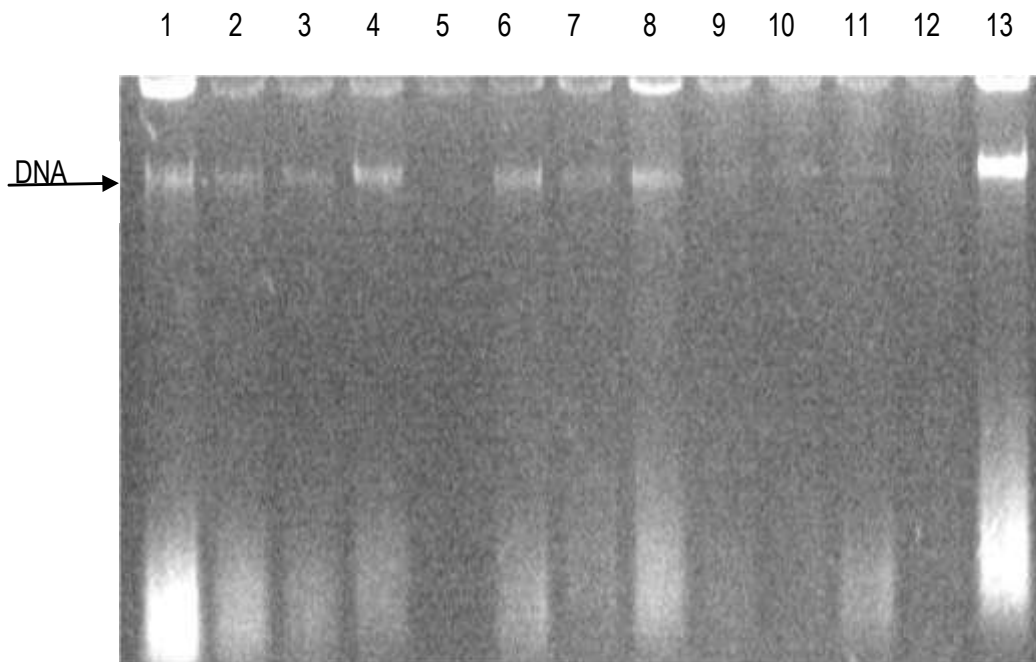
Figure 4. 2: DNA amplification of total Nucleic Acid samples 1-13, showed that the extraction method was good but the samples had different concentrations

4.1.4 Multiplex Polymerase Chain Reaction

Detection and differentiation of *E. histolytica* and *E. dispar* in 169 stools samples performed simultaneously in a PCR cycle. The multiplex PCR performed using the samples initially preserved at -20°C was able to detect and differentiate the species at once rather performing analysis twice (one for detection and another one for differentiation). The presence of bands at 96bp indicated as positive for *E. dispar* and 132bp indicated as positive for *E. histolytica*. Bands not within the 96bp and 132bp makers were indicated as unknown DNA (Figure 4.3).
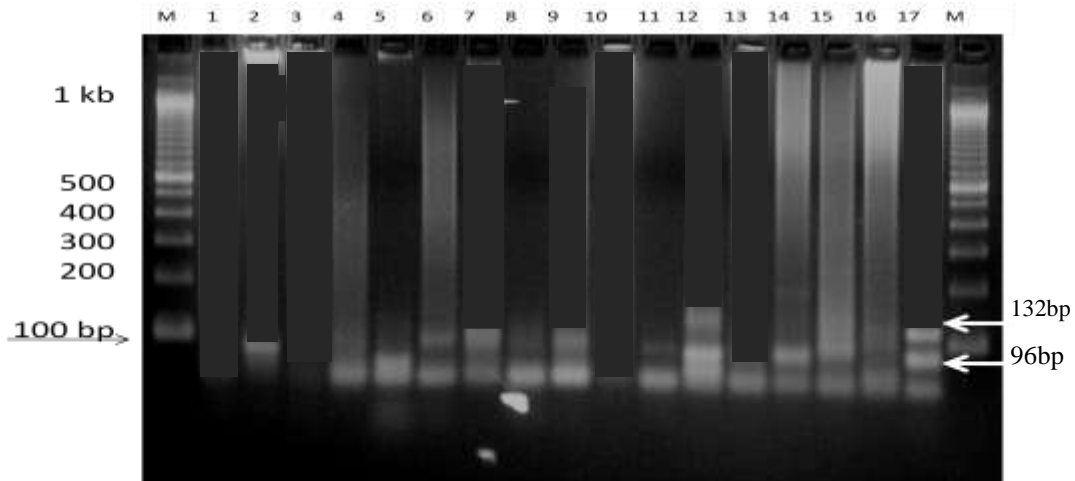
Figure 4. 3: Agarose gel of PCR products amplified by *E. histolytica* primers (EHP1 \ EHP2) and *E. dispar* primers (EDP1 \ EDP2). Molecular ladder/marker size 100bp (M), negative control (lane 1), *E. dispar* positive control (lane 2), *E. dispar* positive samples (lane 6, 7, 9, 11, 14), *E. histolytica* positive control (lane 12), mixed infection with *histolytica* and *E. dispar* (lane17), negative or unknown DNA patient samples (lane 3, 4, 5, 8, 10, 1315, 16).

A comparison of microscopy and multiplex PCR in detection of *E. histolytica and E. dispar* reveals that only 8 of the 169 (4.7%) stool samples contained DNA of *E. histolytica* while 34 (20.1%) contained that of *E. dispar*. In addition, of the 36 samples that had been positively identified for *Entamoeba* species by microscopy and subjected to multiplex PCR, only 6 (16.7%) were found to contain *E. histolytica* DNA while 27 (75%) samples had DNA of *E. dispar* and 3 (8.3%) had unknown DNA. On the other hand, of the 133 samples which were found negative by microscopy, only 2 (1.5%) contained *E. histolytica* DNA while 7 (5.3%) contained DNA of *E. dispar* and 124 (93.2%) contained unknown DNA (Table 4.1).

Table 4.1: Detection rates by Microscopy and Multiplex–PCR

Microscopic examination		Multiplex –PCR	
		E. histolytica	*E. dispar*
Positive	36	6	27
Negative	133	2	7
Total	169	8	34

The Pearson Chi-Square was conducted to test the independence of the variables. There was a significant relationship between microscopy test and multiple PCR test at $\chi 2$ (1df, n=169) =

14.444, p<0.05. On cross tabulation of tests by microscopy and results of tests by multiplex PCR for both *E. histolytica* and *E. dispar* the following were observed. Out of 133 samples found to be negative by microscopy 2 (1.5%) were positive by multiplex PCR for *E. histolytica* and 7 (5.3) %) were positive with *E. dispar*.

4.1.4 Incidence Rates

Mono infection rate with *E. dispar* was demonstrated in 34 out of 169 stool samples (20.1%), while co-infection rate with *E. histolytica* and *E. dispar* was demonstrated 4 out of 169 stool samples (2.4%). Similarly, mono-infection rate with *E. histolytica* was demonstrated 4 out of 169 stool samples (2.4%). The relationship between age, gender and location in comparison to the incidences of the two species showed that there was no significant relationship (Table 4.2).

Table 4.2: Relationship between age, gender and location in comparison to the incidences of *E. Histolytica* and *E. dispar*

Variables	F-value	p-value	Sig.
Age vs. *E. histolytica* and *E. dispar*	0.64	0.960	Ns
Gender vs. *E .histolytica* and *E .dispar*	0.18	0.669	Ns
Location *E .histolytica* and *E. dispar*	1.37	0.200	Ns

Ns= not significant at $p<0.05$, there was no significant relationship between the incidences of *E. histolytica* and *E. dispar* across ages, gender and location.

4.1.5 Sensitivity and specificity of the two techniques

The sensitivity and specificity of microscopy was 73.3% and 98.2% respectively, while for multiplex PCR the sensitivity and specificity was 93.3% and 100% respectively.

Table 4.3: Multivariate tests for sensitivity between microscopy and multiplex PCR tests

Effect		Value	F	Hypothesis df	Error df	Sig.
Level	Wilks' Lambda	0.848^a	14.959^b	2.000	167.000	0.000

a. Design: Intercept

 Within Subjects Design: Level

b. Exact statistic

Multivariate analysis was conducted to compare the sensitivity between multiplex polymerase chain reaction (MPCR) for both *E. histolytica* and *E. dispar* and microscopy conditions. There was a significant effect of sensitivity (Wilks' Lambda = 0.848, F (2,167) = 14.959, p =0 .000). This suggests that MPCR type is more sensitive in differentiating the two species.

4.2 Discussion

Microscopy has remained the gold standard tool for diagnosis of intestinal amoebiasis infection. The recognition of *E. dispar* as new non-pathogenic species which is morphologically indistinguishable from the pathogenic (*E. histolytica*) has necessitated the search for alternative methods for differentiating species within the *Entamoeba* complex (WHO, 1997). The primary advantage of using PCR as a diagnostic tool is the possibility of differentiating the species within the complex (*E. histolytica* and *E. dispar*) in an area where other *Entamoeba* species are prevalent. In addition, PCR has the advantage of accuracy and reliability especially when there is need to understand the epidemiology of *E. histolytica* and *E. dispar* infections. Observations in the current study indicate that diarrhoea and other gastrointestinal infection symptoms appeared to be associated with *E. histolytica* and *E. dispar* infection. However, the cause-and- effect relationship of *E. histolytica* and *E. dispar* with the clinical symptoms could not be determined in this present study due to the limitation of the design given that there was no attempt to rule out other bacterial and/or viral infections. Besides, it has been documented that not all *E. histolytica* infections lead to clinical disease (Ali *et al.,* 2008) which complicate the scenario for drawing conclusion. The finding in this present study showed that all patients who were diagnosed with positive for *E. histolytica* had clinical symptoms of amoebiasis.

The findings of the present study appeared to be consistent with other studies which showed that multiplex PCR is superior than microscopy and the two species are genetically different (Nunez *et al.,* 2001; Santo *et al.,* 2007; Aseel and Sarmad, 2010; Mona *et al.,* 2011). The mono infection rate with *E. dispar* was higher compared to *E. histolytica* while co-infection rate with both species was low. These findings underscore the need for proper diagnosis before administration of *Entamoeba* treatment as per the requirements by WHO, (1997). Only few cases required anti amoeba treatment while those patients from whom the stool contained DNA of *E. dispar* and unknown DNA required further investigation to rule out other causes of gastrointestinal infections.

The present study demonstrated that the incidence of *E. dispar* was higher compared to *E. histolytica* by multiplex PCR. These results are consistent with other studies which reported such observations for example: Gonin and Trudel (2003) found the incidences of the two species as follows: 2 *E. histolytica* and 66 *E. dispar*, Visser *et al* (2006) 6.7% *E. histolytica* and 91.2% *E. dispar*, Fotedar *et al.,* (2007b), 5 *E. histolytica* and 15 *E. dispar* and Aseel and Sarmad, (2010), 26% *E. histolytica* and 43% *E. dispar*. All these studies reported higher incidences of *E. dispar* than *E. histolytica*. These results suggest that other species of

Entamoeba could be present, but in the current study, it was not possible to identify them because the primers used were specific to amplify only DNAs of *E. histolytica* and *E. dispar*. However, a follow up study could concentrate on separation of the *Entamoeba* species with an aim of deciphering the identity of unknown *E. histolytica* species. However, those patients presenting with gastrointestinal problem should also be investigated further for other causes of gastrointestinal infections; this include bacterial and viral. The study, also demonstrated that there was no relationship between the incidences of *E. histolytica* and *E. dispar* across ages, gender and location (Table 4.2). This means the two species affect all patients irrespective of their ages, gender or location. A differential characterization of *E. histolytica* from other intestinal protozoa is essential because only *E. histolytica* infection requires a specific drug treatment. The indiscriminate use of such drug can induce development of drug resistance (Santo *et al.,* 2007).

A comparison of the findings of the two techniques showed that there were some variations: multiplex PCR detected more patients were infected with *E. histolytica* or *E. dispar* compared to microscopy. Some samples which were positive by microscopy turned negative when they were analysed by multiplex PCR. These variations in the results were less, in consistent with other studies where by microscopy examination showed positive for *E .histolytica/E. dispar* complex but negative by multiplex PCR. In Cuba the study showed that 49 out of 52 (94.2%) (Nunez *et al.,* 2001), Brazil, 11 out of 27 (40.7%) (Santo *et al.,* 2007), Iraq, 69 out of 76 (90.8%) (Aseel and Sarmad, 2010), Malaysia, 63 out of 93 (67.7%) (Anuar *et al.,* 2013).

There was some discrepancy between microscopy and multiplex PCR results in 3 samples that had been positively identified by microscopy. However, these PCR did not amplify DNA of the 3 samples, hence these were reported negative yet *E. histolytica* or *E. dispar* with the primers used and there had no inhibition of PCR was observed in control experiments during optimization of multiplex PCR. These negative results can potentially be explained by the presence of other members of *Entamoeba* species complex such as: (*Entamoeba. moshkovskii, E. polecki, E. coli,* and *E. hartmanni*) these discrepancies have been observed by other scholars: in Brazil out 16 out of 27 (59.3%) (Santo *et al.,* 2007), Iraq, 7 out of 76 (9.2%) (Aseel and Sarmad, 2010), Malaysia, 30 out of 93 (32.3%) (Anuar *et al.,* 2013). Another likely reason for this could be the fact that the three samples contained trophozoites that could have degenerated with time during storage. However, in the current study the storage was reduced in order to minimise chances of degradation. To rule out whether lack of amplification was due to inhibitors the three samples were retested using

multiplex PCR and all were negative, Afterwards, the DNAs of the three negative samples were spiked with 0.05μl DNA of the positive controls retested then they all became positive. No evidence of inhibition was found in any of the multiplex PCR negatives. This suggested that other species of *Entamoeba* (*moshkovskii, E. polecki, E. coli,* and *E. hartmanni*) are present in Naivasha. These species were reported in areas of Ghana, Pondicherry and Bangladesh (Santo *et al.,* 2007). However, this supposition needs to be verified further in subsequent studies and probably using a much larger sample size to confirm the presence of other species commonly found in humans.

In addition, the current study further observed that 9 stool samples (8.8%) which were negative by microscopy became positive by multiplex PCR and 2 (1.5%) were found to have the DNA of *E. histolytica* and 7 (5.3%) had that of *E. dispar*. Generally multiplex PCR technique reduced the chances of misdiagnosis by 9 (6.7%) patients. These findings were in agreement with other studies which reported the use of multiplex PCR increases the sensitivity of PCR techniques and allows for simultaneous differentiation between *E. histolytica* and *E .dispar* in a single PCR step as well as the presence of mixed infections (Mona *et al.,* 2011). The Pearson Chi-Square was conducted to test the independence of microscopy and multiplex PCR test. There was a significant relationship between microscopy test and multiple PCR test at $\chi2$ (1, N=169) = 14.444, p<0.05. On cross tabulation of tests by microscopy and results of tests by multiplex PCR for both *E. histolytica* and *E. dispar* the following were observed. Out of 133 samples found to be negative by microscopy 2 (1.5%) were positive by multiplex PCR for *E. histolytica* and 7 (5.3) %) were positive with *E. dispar* a total of 9 (6.8%) samples were positive by multiplex PCR (Table 4.1). These findings show that the multiplex PCR is more sensitive and should be encouraged as technique for diagnosis of amoebiasis. These will reduce the false negative results under microscopy and correct diagnosis will be achieved. These findings provide important data for public health care system in Kenya because this is the first time the species of the *E. histolytica/E. dispar* complex circulating in this country have been differentiated at DNA level. This will reduce the cost of treatments to patients who are diagnosed with non pathogenic species *E. dispar*.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The study showed that multiplex PCR is a robust procedure and easily adapted to routine use and can serve as a tool for detection and confirmation of microscopy results. It is also useful tool for distinction between *E. histolytica* and *E. dispar* as well as the presence of mixed infection simultaneously in a single PCR round in laboratory analyses. To the best of my knowledge, this is the first report that has differentiated *E. histolytica* and *E. dispar* from human faecal samples from Kenya. The incidence of *E. dispar* was highest compared to *E. histolytica* while there were only a few cases of mixed infection. The sensitivity and specificity of multiplex PCR was 93.3% and 100% respectively compared to microscopy 73.3% and 98.2% respectively. Further investigations are essential for those patients harbouring *E. dispar* to rule out other causes of gastrointestinal infections example bacterial or viral infection. However, multiplex PCR technique does not substitute the microscopy stool examination which widely screens for virtually intestinal parasite but may be a useful tool for diagnosis and differentiation and epidemiological studies in areas where *E. histolytica* is endemic. Despite cost of carrying out the technique being higher compared to conventional microscopy, multiplex PCR is more sensitive and specific in detection and differention *E. histolytica* and *E. dispar*. However, further epidemiological survey and clinical studies are needed to determine the true pathogenic potential of the two species.

5.2 Recommendations

1. The government, through Ministry of Health in Kenya, should make a new policy in *Entamoeba* diagnosis. No patient should be treated using microscopy results alone. All positive *Entamoeba* cases must be confirmed using multiplex PCR before one is given anti-amoeba drugs. Even though the cost of drugs ranges between Ksh250-Ksh400, this will reduce the chances of resistance of currently recommended anti-amoeba drugs.

2. More epidemiological surveys should be done in the whole country so that the actual prevalence of *Entamoeba species* can be established.

3. The government should procure PCR equipment and reagent in each of county referral hospital where specimens which are positive by microscopy can be referred for differention of the two species at Ksh 400.

4. Microscopy should be used as screening technique for amoebiasis but multiplex PCR techniques used for differentiation, so that one can confirm whether one has mono infection or mixed infection at Ksh 250.

5. Those patients with gastrointestinal symptoms, but microscopy results negatives and multiplex PCR doesn't detect the amoeba DNA, further tests are required to rule out other causes of gastrointestinal infections such as protozoan, helminthes, bacterial and viral infections.

6. The government, through ministry of health should ensure all sewerage systems are treated and water sources protected to avoid being contaminated with sewerage.

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APPENDICES

APPENDIX I: CONSENT FORM

My name is Samuel Gutu Gachuhi an MSc Medical Parasitology student undertaking Medical Parasitology course at Egerton University. I am carrying out my study in Naivasha district hospital.

The purpose of this study is to identify and distinguish the pathogenic *E, histolytica* and non pathogenic *E.dispar* species from patients seeking medical attention in outpatient department with clinical symptoms of amoebiasis. Finding of this study will determine the incidences of *E. histolytica* and *E.dispar* for proper case management of amoebiasis.

For the study to success, I will require stool specimen from you and the laboratory result will be confidential, though it will be used anonymously to give other stakeholders. Result will not be given to you but if you wish to know the finding you may contact the researcher on cell phone 0723939765.

No pain or harm will be inflicted on you as the study is carried out. Participation is voluntary, hence you have a right to decline and if decline this will have no negative effect on you. There is no cost implication on your part and no incentive will be issued to you.

I..agree to voluntarily take this part in this study

Sign..
Date..
Witness sign..
Date..

APPENDIX II: EGERTON ETHICAL CLEARENCE

EGERTON

TEL: 051-2217808
FAX: 051-2217942

UNIVERSITY

P. O. BOX 536
EGERTON, KENYA

OFFICE OF THE DEPUTY VICE-CHANCELLOR
DIVISION OF RESEARCH & EXTENSION

RESEARCH ETHICS COMMITTEE

Ref: EU/DVRE/028

December 6, 2012

Gachuhi Samuel Gutu
P.O. Box 536,
EGERTON

RE: APPLICATION FOR ETHICAL APPROVAL OF RESEARCH PROJECT

Reference is made to your application for ethical clearance of your research project entitled
**"Differentiation of *Entamoeba Histolyca* and *Entamoeba Dispar* Complex by Multiplex
Polymerase Chain Reaction.**

This is to inform you that the Egerton University Research Ethics Review Committee met on 4th
Dec. 2012 and discussed your application.   The Committee observed that due consideration
was given to the following ethical issues that would arise from the conduct of the study:

i)   That participation is based on informed consent from participants.
ii) That the specimen collection and tests is a routine activity done in many hospitals and
     therefore the participants are not exposed to risks.
iii) That prior consent shall be given by participants involved in the study
iv) That samples would be coded and therefore the identity of the participants would not be
     exposed to the public.
v)   That the study is beneficial as participants found to be positive with parasites would be
     treated.

The committee therefore gave ethical clearance to your   research project. Please further note
that the Standard Operating Procedures (SOPs) requires that   you submit a copy of the final
report of your study to the Committee.

Prof. M. K. Limo
CHAIRMAN – RESEARCH ETHICS COMMITTEE

c.c. DVC (R&E)
      Director Research

MKL/pao

Egerton University is ISO 9001 : 2008 Certified

APPENDIX III: CLEARENCE LETTER FROM THE MINISTRY OF MEDICAL SERVICES

MINISTRY OF MEDICAL SERVICES

Telegrams Medical –Naivasha

Telephone: 050 - 2020053
            050 - 2020576

DISTRICT MEDICAL SERVICES OFFICE
NAIVASHA DISTRICT HOSPITAL
P. O. BOX 141
NAIVASHA

Our Ref. NVA/ADM/7 VOL.II/148

28th December 2011

Samuel Gutu Gachuhi
SM 17/2841/10
P O Box 3790- 20100
NAKURU

RE: RESEARCH PROJECT

Following your application to carry out research project on Entamoeba histolytica/dispar on patient with case definition of amoebiasis, the Research Ethical Committee has approved your research.

The research period is from January 2012 to April 2012 and any extension will need to be considered by the Research Committee.

DR. MBURU J. M.
MEDICAL SUPERINTENDENT
NAIVASHA DISTRICT HOSPITAL