ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF BIOLOGY

Entamoeba species commonly Diagnosed as *Entamoeba histolytica* and Enteric Bacteria in the Pathogenesis of Diarrhea in Addis Ababa.

By

Asnakew worku

In partial fulfillment of the requirement for the attainment of the degree of Master of Science in Biology (Biomedical Sciences)

Addis Ababa

August, 2007

Acknowledgment

First and foremost I give thanks and praise to my Lord and Savior Jesus Christ for being my rock during this entire process. He has given me more blessings than I deserve. My deepest gratitude goes to my supervisors, Prof. Beyene Petros and Dr. Amha Kebede for their valuable support and encouragement. The inputs they provided at various phases of this work and through correcting the thesis were very valuable. Special thanks go to Dr. Amha Kebede, for his help from the beginning to the end of this project, which was very substantial. I am very much indebted for his maximum cooperation for providing me what he has and for his encouragements and friendly approch. I have had a good fotune to work with him.

I am very much thankful to members of the department of Parasitology, Bacteriology, and serology at EHNRI for their substantial help during the lab work especially to Ato Indris Mohammed, Ato Hussein Mohammed, Ato Kidanemariam Mammo, Ato Suraffel, Dr. Tekola Endeshaw, Ato Gemechu Tadesse, Ato Markos Silesh, Ato Birhanu, W/ro Zewdnesh, W/ro Zewdie, W/ro Askale, W/t Mulu Girma, W/t Mulu-Shiwa and all other members of the staffs. Also, I would like to thank physicians and members of Laboratory department at Selam Health Center and Addis Ketema Propride Clinic namely, Dr. Daniel, Dr. Etefa, Dr. Solomon, Ato Melkie Sharew, Ato Tariku Bekele, Ato Mulatu Kassye, Bekele, W/ro Marta Hailemariam, W/t Hiwot Ketema, and W/t Mulu Kelkay. The contribution of Ato Indris Mohammed, Hussein Mohammed was very substantial.

Appreciation is also extended to the Ethiopian Health and Nutrition Research institute, AAU's Graduate Program and Department of Biology especially to Dr. Dawit Abate and Dr. Gurgia Belay. I would like also to thank the Amhara national regional state education Bureau for giving this previlage. This study could not have been possible without the cooperation of the study participants, health management staff in the study sites.

Lastly, I want to say thanks to my families and friends for their prayers, courages and moral and material support during the entire process. I had so many of helpful people to mention few W/ro Hiwot Birhanu, Ato Assefa Wubalem, Ato Desta Birhane, Ato Anbessie Zewudu, Ato Mulugeta Redae, W/t Yemisrach Zewdu, W/t Aster Wale, Ato Tibebu Fikru, Ato Solomon Getahun, and Ato Adane Birhane.

Table of Content

Contents……....page

ACKNOWLEDGMENT I

LIST OF TABLES V

LIST OF FIGURES VI

LIST OF ABBREVIATION VII

1. INTRODUCTION 1

1.1. AMOEBIASIS 1
1.2. HISTORICAL BACKGROUND 2
1.3. BIOLOGY AND LIFE CYCLE 4
1.4. CLINICAL MANIFESTATION 6
1.4.1. Asymptomatic Colonization 6
1.4.2. Amoebic dysentery/ amoebic colitis 7
1.5. CLINICAL DIAGNOSIS OF AMOEBIASIS 9
1.6. LABORATORY DIAGNOSIS OF AMOEBIASIS 12
1.6.1. Microscopy 12
1.6.2. Culture and isoenzyme analysis 13
1.6.3. Serology 13
1.6.4. Antigen detection tests 14
1.6.5 Molecular diagnosis 15
1.7. EPIDEMIOLOGY 16
1.8. STATEMENT OF THE PROBLEM 17

2. OBJECTIVES OF THE STUDY 19

2.1 GENERAL OBJECTIVE 19
2.2 SPECIFIC OBJECTIVES 19

3. MATERIALS & METHODS 20

3.1. THE STUDY POPULATION 20
3.2. SAMPLE COLLECTION 20
3.3. MICROSCOPIC DETECTION OF PARASITE 21
3.4. DNA EXTRACTION AND PCR AMPLIFICATION 21
3.5. *E. HISTOLYTICA/E. DISPAR* SPECIFIC ELISA 22
3.6 .*E. HISTOLYTICA* SPECIFIC ANTIGEN ELISA 23
3.7. ISOLATION AND CHARACTERIZATION OF ENTERIC BACTERIA 23
3.7.1. Isolation of Salmonella and Shigella spp 24
3.7.2. Isolation of Escherchia coli 24
3.8. ANTIMICROBIAL SUSCEPTIBILITY TEST 24
3.9. DATA ANALYSIS 24
3.10. ETHICAL CONSIDERATION 25

4. RESULTS 26

4.1. CLINICAL HISTORY 26
4.2 MACROSCOPIC AND MICROSCOPIC EXAMINATION OF THE STOOL SAMPLES 26

4.3 MICROSCOPIC DIAGNOSIS OF *E. HISTOLYTICA /E.DISPAR* AND OTHER INTESTINAL PARASITES 27
4.4 COMPARISON OF MICROSCOPY WITH PCR BASED METHOD 31
4.5. *E. HISTOLYTICA/E. DISPAR* SPECIFIC ATIGEN DETECTION BY ELISA 31
4.6. ENTERIC BACTERIA CULTURE 33
4.7. ANTIBIOTIC SUSCEPTIBILITY TEST. 39
5. DISCUSSION 41
6. CONCLUSIONS 49
7. RECOMMENDATIONS 50
8. REFERENCES 51
9. APPENDIXES 60

List of Tables

Table 1. Comparive features of amoebic & bacillary dysentery with clinical symptoms and stool findings of patients……11
Table 2. Prevalence of intestinal parasites among the 150 diarrheal patients diagnosed by direct microscopy and formol-ether concentration method from Selam Health Center and Pro-pride Clinic, Addis Ababa , December, 2005-April, 2006……..28
Table 3.Diagnosis of *E. histolytica/ E. dispar* by Microscopiy and the polymerase chain reaction (PCR) method in relation to the clinical history of diarrheal patients at Selam Health Center and Propride Clinic, Addis Ababa December, 2005-April, 2006……...29
Table 4. Microscopic finding of trophozoites of *Entamoeba* spp. in the study participants co-infected with other parasites and diarrheagenic bacterial pathogens isolated by standard culture among 150 diarrheal patients diagnosed in Selam Health Center and Propride Clinic, Addis Ababa December, 2005- April 2006……..……...30
Table 5.Comparison of microscopic and species specific PCR based method for diagnosis of *E. histolytica/E. dispar* among 150 diarrheal patients diagnosed at Selam Health Center and Propride Clinic, Addis Ababa, December, 2005-April, 2006……31
Table 6. Comparison of *E. histolytica/E. dispar* specific antigen detection ELISA with the PCR based method among 150 diarrheal patients diagnosed at Selam Health Center and Propride Clinic, Addis Ababa December, 2005-April 2006……..…32
Table 7.The sero-group prevalence of Shigella isolates among 150 diarrheal patients from Selam Health Center and Propride Clinic, Addis Ababa, December, 2005- April 2006……...34
Table 8. The association of clinical history and stool findings with the isolation of bacterial pathogens (*Shigella* spp.) among 150 diarrheal patients at Selam Health Center and Propride Clinic in Addis Ababa, December, 2005-April 2006…….36
Table 9.The relative frequency of *Shigellae* and *E. histolytica/E.dispar* trophozoites in relation to the clinical history of patients at Selam Health Center and Propride Clinic, Addis Ababa, December 2005-April 2006……..38
Table 10.The relative frequency of *Shigellae* and *E. histolytica/E.dispar* trophozoites in relation to the stool findings of patients at the time of diagnosis at Selam Health Center and Propride Clinic, Addis Ababa, December 2005-April 2006……39
Table 11. The antibiotic resistance pattern of *Shigella* spp. isolated from Selam Health Center and Prop ride Clinic in Addis Ababa by disc diffusion method (December 2005-April 2006)……40

List of Figures

Figure 1.The life cycle of *E. histolytica* and clinical manifestations in humans (Huston *et a l*., 1999). 5
Figure 2. Agarose gel separation of PCR-amplified products of the short tandem repeating units of tRNA genes of *E. histolytica* and *E. dispar*: 1(100 base pair marker), 2 (*E. histolytica* HM1 positive control), 3(*E. dispar* SAW positive control), 5-6(*E. dispar*)…….....32
Figure 3.Appearance of *Shigellae* on MacConkey agar 33
Figure 4. The distribution of *Shigella* spp.with regard to age among the 150 diarrheal patients by bacteriological method from Selam Health Center and Propride Clinic, Addis Ababa, December, 2005-April, 2006……..37

List of Abbreviation

ALA	=	Amoebic Liver Abscess
ATP	=	Adenosine triphosphate
BP	=	Base Pair
CIE	=	counter immuno electro phoresis
DNA	=	Deoxyribose Nucleic Acid
dNTP	=	Deoxyribose nucleotide triphosphate
EHNRI	=	Ethiopian Health and Nutrition research institute
ELISA	=	Enzyme linked immunosorbent assay
Gal	=	galactose
IFA	=	indirect immuno fluorescent assay
IgA	=	immunoglobulin A
IgG	=	immunoglobulin G
IgM	=	immunoglobulin M
IHA	=	Indirect haemagltination
Kb	=	Killo base
KD	=	kilo dalton
MAC	=	MacConkey Agar
Nac	=	N-acetyl galactosamine
Nm	=	nano-meter
OD	=	Optical density
PCR	=	Polymerase chain reaction
PVPP	=	Polyvinyl polypyrolidone
RBC	=	red blood cells
RPM	=	revolutin per minute
rRNA	=	ribosomal RNA
SAF	=	Sodium acetate acetic acid formalin
SSA	=	Salmonella shigella agar
SSrRNA	=	Smaller sub-unit ribosomal ribonucleic acid
STR	=	short tandem repeats
tRNA	=	transfer RNA
Ml	=	Micro-lter
Mm	=	micromete
WBC	=	white blood cells
XLD	=	xylose lysine desoxycholate

Abstract

Clinical reports relying on microscopic diagnosis give an impression that intestinal amoebiasis is very common in Ethiopia. However, recent species specific PCR-based method had shown little or no true infection with *Entamoeba histolytica*. The present study was conducted to assess the association of diarrheagenic bacteria vis-à-vis the *Entamoeba* trophozoites commonly diagnosed as *E. histolytica* in diarrheal patients. One hundred and fifty diarrheal patients from Selam Health Center in Gulelie Sub-city and Addis Ketema Propride Clinic in Addis Ababa were recruited in a study conducted between December 2005 and April 2006. Single stool samples were collected from the study participants, and inspected macroscopically for consistency, appearance, and the presence of gross blood. Wet mount of the stool samples was made to detect the presence of fecal leukocytes and red blood cells (RBCs*)*, *E. histolytica/E. dispar* trophozoites and other intestinal parasites. The specimens were further tested for the presence of *E. histolytica/E. dispar* DNA by PCR; for stool antigens of *E. histolytica* by antigen ELISA; and by stool culture for dirrheagenic bacteria. Microscopic observations revealed *E. histolytica/E. dispar* to be the most common parasite diagnosed (40.7% of the diarrheic patients) followed by *Blastocytis hominis* (24.7%) and *Giardia lamblia* (12.7%). However, the PCR based method which amplifies short tandem repeating gene sequence on the tRNA confirmed only 10 cases (16.4%) of *E. histolytica/E. dispa*r. Out of these only two (3.3%) were the pathogenic *E. histolytica* .The *Entamoeba* specific stool antigen ELISA detected 30 cases (20%) to be *E. histolytica /E. dispar.* However, only two of the ELISA positive cases were confirmed by the PCR based method. Of the 150 diarrheal patients, 57(38%) were Shigellae and 1(0.4%) *Escherichia coli B.* Infection with trophozoites of *Entamoeba* species showed no clear association with clinical symptoms such as history of bloody-mucoid stools, history of low-grade fever, and microscopic finding of pus cells and RBCs($P>0.05$). On the other hand, the isolation of *Shigellae* in patients with these clinical symptoms was significantly higher ($P<0.05$). High rate of co-infection in patients with trophozoites of *Entamoeba* spp. were observed most commonly with *Shigella* spp. (41.3%), *Blastocystis hominis* (32.8%), *Giardia lamblia* (14.8%). Consistent with the previous reports, the present study indicated the rare occurrence of *E. histolytica* in Ethiopia and the inadequacy of microscopy for diagnosis of amoebiasis. The finding in this study reinforces the suggestion that patients with enteric bacterial pathogens may be wrongly diagnosed as suffering from amoebiasis simply due to the presence of non-pathogenic amoeba trophozoites in their stools. However, the contribution of enteric coccidians, like *Cryptosporidium parvum*, *Isospora belli,* and *Microsporidia* must also be assessed to more correctly establish the etiologyof diarrhea in Ethiopia.

Key words: *Entamoeba histolytica, E. dispar,* entreic bacteria, co-infection, PCR, ELISA

1. Introduction

1.1. Amoebiasis

Amoebiasis is an infection of the human gastrointestinal tract by *Entamoeba histolytica*, a protozoan parasite that is capable of invading the intestinal mucosa and that may spread to other organs, mainly the liver. It is considered as one of the major health problems in tropical and sub-tropical areas. It is characterized by low socio-economic status and poor hygiene that favours the indirect fecal-oral transmission of the infection. Amoebiasis is estimated to cause 50 million infections resulting up to 100, 000 deaths per anum (WHO/PAHO/UNESCO, 1997). Thus, it ranks second only to malaria as cause of death resulting from protozoan disease alone. Earlier epidemiological studies based on microscopic examination of the stool for *Entamoeba* trophozoites and cysts showed that approximately 500 million people (10% of the World population) are found to be parasitized with *E. histolytica* (Walsh, 1986). However, based on biochemical, immunological, and genetic data, *E. histolytica* has been reclassified into two morphologically identical but genetically distinct species: *E. histolytica*, which is potentially invasive, and *E. dispar*, which is not (Diamond & Clark, 1993).

At the experts meeting in Mexico, it has been recommended that *E. histolytica* should be specifically identified and if present treated. However, there are still no inexpensive and practical diagnostic procedures available for specific identification of *E. histolytica* at health center level (Leiva *et al*., 2006; Kebede, 2005). There are also large numbers of species of *Entamoeb*a, which parasitize the human intestinal tract. However, only *Entamoeba histolytica* is associated with intestinal and extra intestinal disease. Occurrences of these *Entamoeba* trophozoites, though they are non-pathogenic, have diagnostic challenge. Differential diagnosis of amoebic and bacillary dysentery is the other difficulty as the two diseases present similar clinical symptoms.

1.2. Historical background

Amoeba was first discovered in 1875 by a clinical assistant, D.F. Losch, in St Petersburg Russia (Smith & Barrette, 2000) from a patient with bloody diarrhoea passing a large number of amoebae in his stool, which he called *Amoebae coli*. Losch was able to successfully infect a dog and observe the associated pathology. However, he did not realize that the amoeba was responsible for dysentery but he considered it as a cofactor for disease progression. Quincle and Roos (Guerrant, 1986) recognized the cyst of amoeba first in 1893. F. Schuadin described the organism formally in 1903(Elsdon-Dew, 1968). He was able to distinguish between the *Entamoeba coli* found in healthy people and erythrocyte engulfing amoeba, which he called *E. histolytica*. On the other hand, in 1919 Dobell came with different conclusion about the existence of one species of *Entamoeba* that produced four nuclei for this organism and he retained Schuadin's name *E. histolytica* (Clark, 1998). Due to the fact that not all the people apparently infected with the parasite develop disease, the existence of parasite with different virulent behavior was suggested.

However, in 1925 Emile Brumpt came up with an alternative explanation that there were in fact two species, one capable of causing invasive disease and one that never cause disease (Brumpt, 1925 cited in WHO/UNESCO, 1997). He named this morphologically identical species as *E. dispar*. The concept was ignored because of the difficulty to prove his hypothesis experimentally at that time. The resurrection of *E. dispar* as distinct species started in 1973 after the publication by Martinez-Palomo, showing difference in agglutination pattern between *E. histolytica* isolated from individuals with disease and from those with asymptomatic infections (Martenz-Palmo *et al*., 1973 cited in Clark, 1998). Later, Sargeaunt & Williams (1978), using isoenzyme electrophoresis, proved the existence of two groups of *E. histolytica*: pathogenic zymodeme isolated from individuals with invasive disease and some asymptomatic carriers, and non-pathogenic zymodeme isolated from asymptomatic carriers but none from amoebic disease. A different idea was forwarded claiming that non-pathogenic strains could be converted into pathogenic zymodemes during axenization (Mirelman *et al.*,, 1986) where the two species are not

genetically distinct. In contrast to this, DNA probes efficient enough to detect subtle differences between homologous genes clearly showed pathogenic and non-pathogenic strains of *E. histolytica* are evolutionarily related but genetically distinct species (Tannich *et al*., 1989).

An increasing number of biochemical, immunological and genomic differences between the two species is now recognized. Thus, such kind of evidences finally led to the formal separation of the two species (Diamond & Clark, 1993) with the name *Entamoeba histolytica* being retained for the pathogenic species and Brumpt's name *E. dispar* being revived for the non-pathogenic strain. Zymodeme analysis from an *Entamoeba* cultured from homosexuals and patients with acquired immuno deficiency syndrome (AIDS) suggested that acute infections with *E. dispar* do not result in detectable morbidity even in AIDS patients (Allonson-Iones *et al*., 1988).

Jacobs and his colleagues in 1988 showed differences in *E. histolytica* and *E. dispar* expression of proteins thought to be involved in virulent behavior (cited in Freitas *et al*., 2004). These proteins include lectins that mediate adherence to the epithelial cells, pore-forming proteins, and secreted proteases that degrade host tissue. As reviewed in Reed *et al*. (1996), DNA probes based on rRNA sequences were shown to be specific for isolates of either *E. histholytica* or *E. dispar*. Sequence variability both in protein coding and non-coding genes has been shown to vary between the two species (Clark *et al*., 2002). These sequence differences are used for molecular based diagnosis of the two species.

The recognition of *E. dispar,* as a distinct species, has profound implications for diagnosis, treatment, and epidemiology of amoebiasis (WHO/PAHO/UNESCO, 1997). This recently identified, morphologically identical species, *E. dispar* accounts for about 90% of the estimated cases of infections (Lucas and Upcroft, 2001), partly accounting for the low incidence of clinical amoebiasis. WHO has recommended that it is not appropriate to treat for asymptomatic individuals with *E.histolyica /E.dispar* unless that *E. histolytica* is specifically identified (WHO/PAHO/UNESCO, 1997).

1.3. Biology and life cycle.

The intestinal parasite *E.histolyica* exists in two different forms: an infective cyst and invasive trophozoites. The trophozoite is the actively metabolizing, mobile stage, and the cyst is dormant and environmentally resistant. The cysts measure 10–15 μm in diameter and typically contain four nuclei. *E. histolytica* cysts are resistant to acidification, chlorination and desiccation. They are capable of surviving in a moist environment for several weeks. Infection is initiated by ingestion of quadrinucleated cysts from fecally contaminated food or water, a common occurrence among the poor in developing countries (Haque *et al*., 2003). Excystation occur in the terminal ileum, with each emerging quadrinucleate trophozoite givingrises to eight uninucleated trophozoites.

The trophozoites may either live as a commensal in the lumen or colonize the large intestine and cause dysentery. They may also spread via the bloodstream and may give rise to extraintestinal lesions, mainly liver abscesses. The detail of the life cycle is shown in fig.1 below. The diameter of the trophozoite varies between 10 and 60 μm; its variability is affected by changes in temperature, pH, osmolarity and redox potential, as well as feeding conditions actively invading amoebae tend to be larger (Martenz-Palmo & Espinosa-Cantenalo, 1998). If amoebas pass down the colon, they encyst under the stimulus of desiccation, and then are evacuated with the stool.

E. histolytica displays an unusual cellular organization, many typical organelles are absent in these cell (Bhattacharya *et al*., 1998). *Entamoeba* trophozoites are opportunistic anaerobes with peculiar glycolytic enzymes found in other amitochondrial eukaryotes. The major metabolic end products are ethanol, CO_2, and acetate whose proportions vary depending on the concentration of oxygen in the immediate environment. The other unique aspect of glycolysis in *E. histolytica* is the utilization of pyrophosphate instead of ATP as an energy source in several glycolytic reactions (Martinez- Palmo & Espinosa-CantellanoCantellano, 1998).

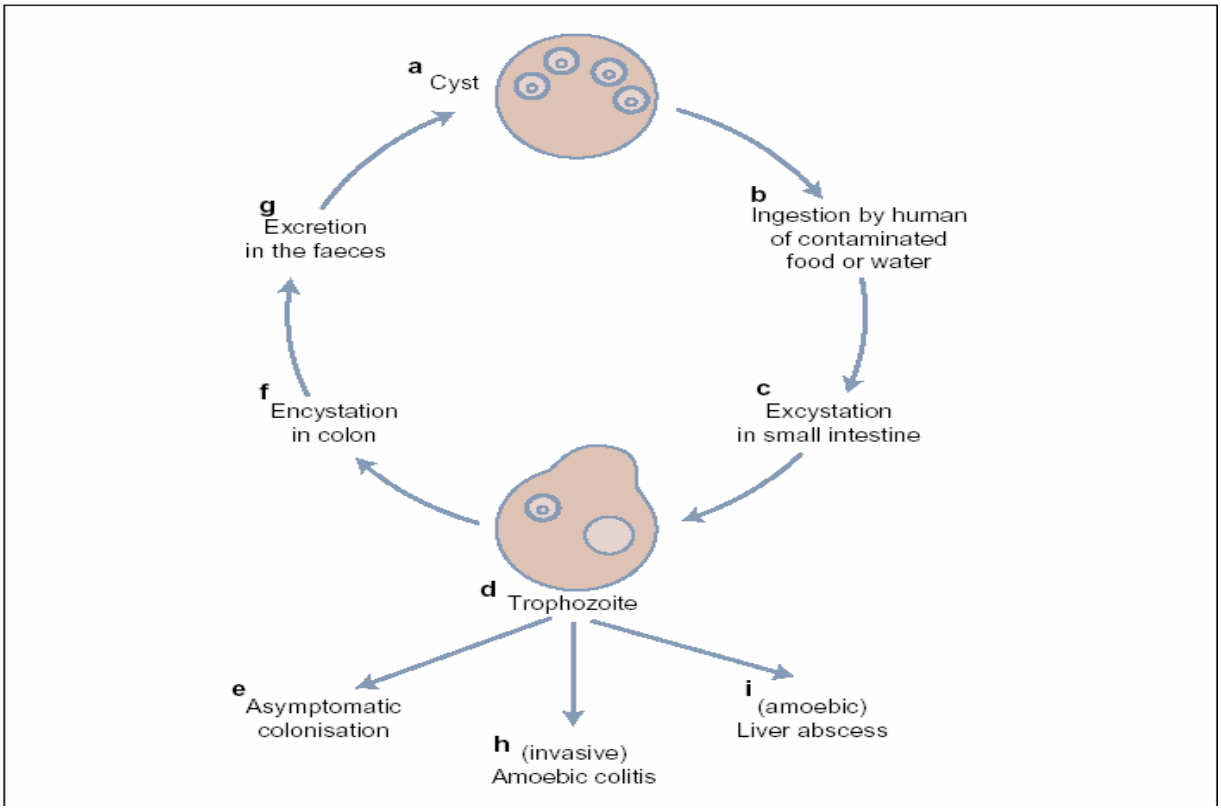

Figure 1.The life cycle of *E. histolytica* and clinical manifestations in humans (Huston *et a l.*, 1999).

The genome of *E. histolytica* is relatively small for eukaryotic (3.2 $X10^7$ bp) and extremely adenine and thymine rich 67% within the coding region and 78 % overall cited in Kebede (2005). The genome of *E. histolytica* also contains multi-copy DNA circles that can easily be cloned in small insert libraries (http://www.sanger.ac.uk/feedback/). The best-characterized circular DNA molecule is the 24.5 Kb episome in strain EhR1 (Bhattacharya *et al*., 1998). It contains two inverted copies of tandemly repetitive rDNA (genes) separated by upstream and down stream inter-genic spacers (Bhattacharya *et al*., 1998).

The restriction map of the *E. dispar* rDNA circle showed close similarity to EhR1, the rDNA circle of *E. histolytica* strain HM-1: IMSS, which has two rDNA units per, circle

(Paul *et al*., 2002). According to the same study, the intergenic spacers in both species were found to have not only similar arrangements but also have close sequence among the two closely related sibling species. Multiple replication origin in these plasmid elements is a noble finding not encountered in any plasmid element (Haque *et al*., 2003). Restriction fragment length polymorphism in rDNA was visible in the first southern blots performed with this DNA (Bhattacharya *et al*., 1998). This polymorphism was attributed to variation of the number in spacer genes. These sequence variation is being utilized to distinguish pathogenic *E. histolyica* from non- pathogenic *E. dispar*.

Another unique feature of *E. histolytica* and *E. dispar* is the existence of short tandem repeats (STRs) located adjacent to most of the tRNA genes (Clark *et al*., 2002; Ali *et al*., 2005). The tandems repeating units varied not only in sequence but also in number and arrangement between the two species at both loci (Zaki *et al*., 2002). Reliable method for PCR-based genotyping of *E. histolytica* based on variation in the numbers of short tandem repeats that are linked to tRNA genes in this species has been developed (Zaki & Clark, 2001; Zaki *et al*., 2002; Ali *et al*., 2005).

1.4. Clinical manifestation

The wide spectrum of intestinal infection ranges from asymptomatic to transient intestinal inflammation or to a fulminant colitis with an array of manifestations that may include toxic mega-colon and peritonitis. The incubation period of intestinal amoebiasis can vary, ranging from a few days to months or years, but is generally 1 to 4 weeks Tanyuksel & Petri (2003).

1.4.1. Asymptomatic Colonization

Asymptomatic infection is characterized by the presence of *E. histolytica* cysts and/or trophozoites in the stool without any symptom of intestinal or extra-intestinal amoebiasis. These patients have normal rectosigmoidoscopic findings, without a history of blood in stool samples, cysts and trophozoites lacking ingested RBCs may be visible on

microscopy Garcia &Brukner (1997). According to Abd-Alla *et al*. (1998) most individuals infected with *E. histolytica*, but not *E. dispar*, develop serum antibody responses to the parasite even in the absence of invasive disease. So far, *E. dispar* has never been recognized as a cause of colitis or amoebic liver abscess, although infection with these amoebae is much more common than with *E. histolytica*, especially in developing countries.

In an attempt to determine the potential invasiveness of asymptomatic infection with *E. histolytica,* Gathiram & Jackson (1987) found that only 10% of those who are infected with *E. histolytica* subsequently developed invasive disease while the majorities (90%) clear their infection within one year. Similar study conducted in Bangladesh showed that only 2 of the 17 children develop amoebiasis, the rest cleared the infection within one year (Haque *et al*., 1999). At present, the diagnosis of intestinal amoebiasis in many countries relies commonly on microscopic examination of stool samples for the presence or absence of *E. histolytica/E. dispar*. Hence, it is not clear what percentage of patients infected with *E. histolytica* are asymptomatic

1.4.2. Amoebic dysentery/ amoebic colitis

Although people can be asymptomatically colonized with *E. histolytica*, they should be treated. Otherwise, some of these subjects, called asymptomatic cyst passers, may develop colitis after a period of months or may be dangerous environmentally in terms of transmission of the disease. The intestinal syndromes caused by *E. histolytica* form a continuum ranging in severity from mild diarrhea to hemorrhagic dysentery.

Patients with amoebic colitis typically present with several-week history of cramping abdominal pain, weight loss, and watery or bloody diarrhea Petri & Singh (1999). Diarrhea can result up to 10 (even more) bowel movements per day, fever occurs in approximately one third of the patients Tanyuksel & Petri (2003). Few patients have low-grade fever, anorexia, nausea, vomiting, weakness, and abdominal tenderness.

The presence of charct-Lyeden crystals, lack of fecal leukocytes, and the presence of blood are the most common stool findings Tanyuksel & Petri (2003). Colonic lesions can vary from mucosal thickening only to flask shaped ulcerations or necrosis of the intestinal wall most commonly in the caecum or appendix or near the ascending colon, but rarely in the sigmoido-rectal area Garcia & Bruckner (1997). The diagnosis of amoebic dysentery should be considered when patients present with microscopically detectable (occult) or grossly bloody diarrhea. However, the insidious onset and the variable nature the symptoms make the diagnosis difficult with fever and grossly bloody diarrhea absent inmost cases (Haque *et al*., 2003). Because of the chronicity of the illness, weight loss is common.

Unusual manifestations of amoebic colitis include acute necrotizing colitis, toxic megacolon, ameboma, and perianal ulceration with potential formation of a fistula. Acute necrotizing colitis is rare (occurring in less than 0.5 percent of cases) and is associated with a mortality rate of more than 40 percent (Haque *et al*., 2003). Patients with acute necrotizing colitis typically appear very ill, with fever, bloody mucoid diarrhea, abdominal pain with rebound tenderness, and signs of peritoneal irritation. Ameboma results from the formation of annular colonic granulation of tissue at a single site or multiple sites, usually in the cecum or ascending colon Juniper (1984). An ameboma may mimic carcinoma of the colon and needs differential diagnosis.

1.4.3. Extraintesinal amoebiasis

After amoebic dysentery, amoebic liver abscess is the second most common form of invasive amoebiasis caused by *E. histolytica.* It arises from haematophagous spread of amoebic trophozoites that have breached the intestinal mucosa probably reaching the portal circulation. Patients with ALA present with 1-2 weeks of fever, right upper quadrant pain Petri (1996). Weight loss, fever, and more diffused abdominal pain, jaundice if ever, occur in the sub-acute phase Tanyuksel & Petri (2003). Unusual sites or complications of extraintestinal amoebiasis include direct extension from the liver to the pleura (and/or pericardium), brain abscess, and genitourinary amoebiasis.

Amoebic liver abscess (ALA) can be a cause of significant morbidity and mortality in all ages, more commonly in the age group of 20-45 years (Mathur *et al*., 2002). However, there are few reports dealing with this entity in extremes ages where it is seven to nine times more common in males (Mathur *et al*., 2002; Sharma & Ahuja, 2003). Amoebic liver abscess occurs in approximately 5-7% of patients who develop amoebic infestation of the intestinal tract (Khotaii *et al*, 2003).

ALA can be challenging to diagnosis as its symptoms are non-specific and develops months or years after the patient leaves endemic areas. All patients with ALA have preceding intestinal infections, but less than 30% have acute diarrhoea at the time of diagnosis Reed (1998). It might mistakenly be diagnosed as pyogenic liver abscess, hepatic neoplasm, and hepatic echinoccocosis (helminthic infection) (Huston *et al*., 1999). As reviewed in Stanley (2003) leukocytosis without eosinophilia, mild anemia, elevated levels of alkaline phosphatase, and high erythrocyte sedimentation rates are the most common laboratory findings. The definitive diagnosis of ALA is confirmed by positive serological tests for antibodies to *E. histolytica* and demonstration of the hepatic lesion by imaging techniques such as computed tomography ultrasonography, magnetic resonance imaging, etc.

1.5. Clinical Diagnosis of Amoebiasis

Clinical diagnosis of amoebiasis is very difficult because of the non-specific nature of the symptoms. The common differential diagnoses include bacterial and viral enteric infections, inflammatory bowel disease, carcinoma of the colon and functional disorders. Organism-specific diagnosis can prevent unnecessary diagnostic procedures or treatments. For example, *E. histolytica* infection in a patient with severe abdominal cramps or bloody stools can prevent unneeded or dangerous colonoscopy, surgery, or corticosteroid treatment for presumed ulcerative colitis.

Shigellae are the causative agent of bacillary dysentery in humans. Colonization of intestinal epithelial cells by this pathogen induces an intense inflammatory reaction that

leads to destruction of the colonic mucosa Sansonetti (1996). The incubation period for *Shigellae* is typically 3 days, with a range of 1 to 7 days. Initial symptoms usually include fever, abdominal cramps, and watery diarrhea, which are followed by abdominal cramps, tenesmus, rectal urgency, and small-volume diarrhea. Bacillary dysentery begins suddenly with fever, chills, severe diarrhea and dehydration, and diarrhea with blood or pus in the stool Jacobson (1979).

Patients with amoebiasis were shown to have relatively longer periods of pre-hospital illness, less frequent fever, lower fecal leukocyte count, and experience weight loss compared to patients with shigellosis (Speelman *et al*., 1987). The findings of this work suggest that patients with amoebiasis seek medical care after an average of 20.8 days of symptoms, where as patients with shigellosis visit hospitals after only 3.9 days. In general, Entero-invasive organisms such as *Shigella* spp., *Campylobacter* spp., and some strains of *E. coli* are characterized by the presence of fecal leukocytes. This suggests an inflammatory colitis with mucosal breakdown. On the other hand colitis caused by enterotoxins secreted by parasites and other strains of *E. coli* show an absence of fecal leukocytes as the diarrhea is secretory. Fecal leukocytes are seen with regularity in symptomatic shigellosis and their presence, though not entirely specific, should suggest an invasive process, which is not characteristic of toxigenic or nonbacterial gastroenteritis (Jacobson, 1979).

The determination of fecal leukocytes by microscopy is a procedure used by many clinical laboratories to identify inflammatory diarrheas caused by enteric bacteria. The presence of one or more WBC per high power field of vision (HPF) was strongly associated with the finding of entero-invasive bacterial pathogens with a sensitivity of 57% in outpatients (Savola *et al*., 2001). The presence of fecal leukocytes more than 5/HPF in stool microscopic examination was significantly more frequent among patients infected with *Shigella* (Khan *et al*., 2006). The presence of fecal leukocytes should suggest a bacterial diarrhea in clinically compatible cases (Savola *et al*., 2001). A recent study in Bangladesh showed direct microscopical examination of stool specimens for the presence of WBC and RBC may facilitate the early diagnosis of shigellosis, and may be a

cheap alternative to stool culture in settings where there are no laboratory facilities (Khan *et al*., 2006). Absence of fecal leukocytes, however, does not exclude bacterial diarrhea or the need for stool culture (http: //www. enh org. / healthandwellnes/encyclopeda/).

Salmonella infections of the intestinal tract produce symptoms similar to those of shigellosis, except the illness may be more chronic, hepatosplenomegaly may occur, and agglutination reactions for salmonella become positive Juniper (1984). The bacterial forms of diarrhea usually cause an inflammatory reaction and small irregular ulcerations in the colon. Viral gastroenteritis tends to be epidemic with an acute onset and often is accompanied by cough, severe nasal congestion, sore throat, muscle pain, headache and vomiting. Idiopathic ulcerative colitis produces diffuse inflammatory changes and irregular ulcerations in the colon, while amoebic ulcers are rounded and appear on an otherwise normal mucosa Juniper (1984).

The presence of *E. histolytica* in the stool does not necessarily indicate that the patient's symptoms are due to the parasite, and the clinical findings must be carefully evaluated Juniper (1984). Amoebic infections also may become symptomatic at a time when another disease is present, thereby masking the latter. For example, carcinoma of the cecum with occult bleeding as its only symptom may go undetected in the presence of acute amoebic colitis. For these reasons, patients with known symptomatic amoebiasis still need stool cultures for pathogenic bacteria, sigmoidoscopy and roentgenographic examination of the colon.

Table 1. Comparive features of amoebic & bacillary dysentery with clinical symptoms and stool findings of patients

Clinical symptom & stool findings	Amoebic dysentery	Bacillary dysentery
Pre-hospital illness	Longer(20.8 days)	Shorter (3.9 days)
Fever	Less frequent	More frequent
Fecal leukocytes	Scanty	Regularly seen
Weigh loss	Common	Rare or absent

1.6. Laboratory Diagnosis of Amoebiasis

Differential diagnosis of *E. histolytica* from the morphologically identical non-pathogenic species, *E.dispar*, is essential for both treatment decision and public health importance (Gonin and Trudel, 2002; WHO/PAHO/UNESCO, 1997). This is mainly due to the wide preponderance of the *E. dispar*, which is non-pathogenic, and needs no treatment at all. Species-specific diagnosis may also give clue for identifying the causative agent responsible for diarrhea other than *E. histolytica*.

1.6.1. Microscopy

Laboratory diagnosis of amoebiasis can be by microscopic identification of characteristic cysts or trophozoites in stool samples. However, microscopic examination has several limitations. More importantly, it can't differentiate *E. histolytica* from *E.dispar* as they are morphologically identical. False positive results from confusing polymorphonuclear leukocytes or macrophages with trophozoites, and cysts of *E. histolytica* can be mistaken for cysts of different species of *Entamoeba*, *Iodoamoeba* or *Endolimax* (Acuna-Soto *et al*., 1993). The fragile nature of trophozoites and delay in the processing of stool samples also affect sensitivity of light microscopy Reed (1998). Many studies have shown that a single stool sample can give positive result in only one-third to one-half suggesting the necessity of serial diagnosis for at least three samples to reduce false positive results (Huston *et al*., 1999).

The finding of haematophagous trophozoites in freshly examined stool samples is a rare occurrence, but it is 100% specific and predictive for invasive amoebiasis (Gonzalez-Ruiz *et al*., 1994). In contrast, the presence of *E histolytica* trophozoites without ingested red blood cells is not a diagnostic indication of active invasive amoebiasis and may reflect the excretion of trophozoites in a patient with diarrhoea of other etiological agent. This brings an implication that species-specific diagnosis cannot be made microscopically unless red blood cell (RBC) engulfing trophozoites are observed.

1.6.2. Culture and isoenzyme analysis

The culturing of stool samples followed by subsequent isoenzyme analysis can accurately distinguish *E. histolytica* from *E. dispar* (Sargeaunt *et al*., 1978). This technique was amongst the earliest to suggest that existence of "pathogenic" and "non-pathogenic" *E. histolytica* as separate species. Until recently it has been applied to more specimens than any other methods and thus deserved its reputation as the "gold standard" against which newer methods need to be validated (Sargeaunt, 1988 cited in Ackers, 2002).

However, it is time consuming, laborious and is not desirable for routine diagnosis of intestinal amoebiasis Reed (1998). More over delays in the processing of the samples, previous-antiamoebic treatments, and the difficulty of culturing cyst positive samples usually, result in false negative results (Huston *et al*., 1999). This has left the laboratory diagnosis of *E. histolytica* infection to exclusively rely upon microscopic examination of fresh and fixed stools especially in developing countries where the disease is endemic.

1.6.3. Serology

Invasive amoebiasis in man may lead to colonic ulcerations, bloody dysentery, and subsequent involvement of internal organs, especially amoebic liver abscess. As reviewed in Huston *et al*. (1999) patients infected with *E. dispar* do not develop serum antibodies, but 75-85% of patients with symptomatic *E. histolytica* infection develop detectable antibodies in the serum acutely. It was also noted that more than 95% of the patients with amoebic liver abscess have serum antibodies to the 170 KD subunit of the galactose inhibitable adherence lectin (Sharma & Ahuja, 2003). The ability of *E. dispar* to induce mucosal antilectin IgA rather than humoral antilectin IgA and IgG has been suggested (Radvin *et al*., 2003). The high titers of antiamoebic antibodies do not show the severity of the disease but to the chronicity of the infection.

The use of antibody detection tests in serum is recommended in the diagnosis of invasive intestinal and extra intestinal amoebiasis. A variety of serological tests are currently

available for the diagnosis of invasive intestinal amoebiasis and its sequel e.g., amoebic liver abscess (ALA). The most common once are indirect haem-agglutination test (IHA), counter immuno electro phoresis (CIE), indirect immuno fluorescent assay (IFA), and enzyme-linked immunosorbent assay (ELISA) (reviewed in Tanykusel & Petri, 2003). These tests may result in both false negative and false positive results as anti-amoebic antibodies developed late after the development of acute infection and persist after the individual is cured of the infection (Huston *et al*., 1999).

Though patients with invasive amoebiasis had high titers of antibody (Radvin *et al*., 2003), the detection of anti-amoebic antibodies was found to be non-specific at titers less than 1:512 using IHA (Pillai *et al*., 1999). The sensitivity for detection of serum antilectin antibody IgM by ELISA in subjects with acute colitis was found to be 45%, compared to 5.6% for antilectin IgG (Abd-Alla, *et al*., 1998). Owing to short life span of IgM compared to IgG, the detection of anti-amoebic IgM may improve the usefulness of serological methods to diagnosis acute disease (Abd-Alla, *et al*.1998; Huston *et al*., 1999). However, the presence of IgG antibodies in a single serum sample does not indicate whether the infection was acquired before or during travel to an area of endemic infection (Pillai *et al*., 1999). Serological methods by detection of serum antibodies against amoebae with crude antigens could be helpful in non-endemic countries where there is no previous exposure. Currently the use of highly purified antigens has improved the use of serological methods.

1.6.4. Antigen detection tests

Detection of galactose inhabitable adherence lectin (GAIP) in the serum using monoclonal antibodies specific epitopes was found to be a highly specific marker for infection with pathogenic *E. histolyitica* (Abd-Ala *et al*., 1993). The target molecule which has been most intensively studied is the heavy subunit of the galactose/N-acetyl-galactosamine inhibitable lectin; of six monoclonal antibodies raised against this molecule (Petri *et al* 1990b), only two reacted with *E. histolytica* and *E. dispar* while the other four reacted only with *E. histolytica* (Petri *et al* 1990a). A monoclonal antibody

prepared against *E. dispar* cultured monoxenically with *Crithidia fasciculata*, was found to specifically react with *E. dispar* isolates, regardless of their geographic origin, the culture conditions, or zymodemes, but did not react with any other enteric protozoan, including *E. histolytica* (Tachibana *et al*., 1997). Tachibana *et al*. (1999) were able to clone and express the genes coding for human antibody specific for *E. histolytica* and indicated the possibility for the production of human monoclonal antibodies specific for *E. histolytica* that can be used in serodiagnosis. These antibodies are the basis of two kits manufactured by TechLab Inc. (www.Techlab.com) one of which (based on one of the non-specific monoclonal antibodies) which identifies *E. histolytica*/*E. dispar* complex while the other, based on a specific one that can identify *E. histolytica*.

Compared with serum antibodies, circulating amoebic antigens do not persist after successful treatment, hence, detection of amoebic antigen in the serum is not only more sensitive than antibody ELISA but it could also show efficacy of antiamoebic treatment (Haque *et al*., 1998). According to the work of Haque *et al*. (1995) enzyme-linked immunosorbent assays that detect amoebic antigen in fresh stool are more sensitive and specific than microscopy when compared with culture as gold standard. They have also shown that the antigen detection tests for *E. histolytica/E. disp*ar complex are as reliable as and much easier than zymodyme analysis for differentiation of *E. histolytica* from *E.dispar*. Irrespective of these reports, other study showed this method was less sensitive than microscopy to distinguish between *E. histolytica* and *E. dispar* (Gonin & Trudel, 2003).

1.6.5 Molecular diagnosis

Current data indicate that *E. dispar* is 10 times more common than *E. histolytica* worldwide (Lucas & Upcrofti, 2001) but local prevalence's may vary significantly. Nevertheless, it is already clear that not all *E. histolytica* infections lead to disease in the host but at most 1 in 10 *E. histolytica* infections progresses to the development of clinical symptoms (Gathiram & Jackson, 1987). Highly specific and sensitive methods to fill the gap of this are required. As reviewed in Huston *et al*. (1999) several polymerase chain

reaction (PCR) based methods that amplify and detect *E. histolytica* DNA in the stool have been developed. The ability of PCR to specifically amplify minute amounts of pathogen DNA has revolutionized the diagnosis of many infectious diseases, and the numerous sequence differences between homologous genes in *E. histolytica* and *E. dispar* make it a natural candidate for identifying these two species.

Polymerase chain reaction (PCR) based on the amplification of amoebic DNA is very sensitive and specific to detect *E. histolytica* and *E. dispar* in human feces. PCR based amplification of ribosomal ribose nucleic acid (rRNA) genes from cultured amoebae trophozoites were found to be 100 fold more sensitive than currently available antibody based methods (Mirelman *et al*., 1997). PCR's ability to detect infection in microscopic negative samples has been noted (Verweij *et al*., 2003; Levi *et al*., 2006). PCR methods rely on sequence differences on homologous genes in *E. histolytica* and *E.dispar* (Acuna-Soto *et al*., 1993; Aguirre *et al*., 1995; Verweij *et al*, 2000; Gonin & Trudel, 2003). Most of these methods rely on amplifying unique regions of the small sub unit rRNA (SSUrRNA) episome. The high copy number provides increased sensitivity. PCR based methods targeting on short tandem repeats (STRs) located adjacent to tRNA (Zaki *et al*., 2002;Ali *et al*., 2005) and cysteine proteinase and serine coding genes (Frietas *et al*., 2004) have been developed. The disadvantage of PCR based methods is that it is time consuming, require skilled personnel, involve multiple steps, and unaffordable for routine diagnosis. Further processing of the amplified products, this may lead to cross contamination and may result in false positive results. Though there are several PCR tests, real time PCR assays utilizing molecular-beacon probe for detection of *E. histolytica* was the most sensitive (Roy *et al*., 2005).

1.7. Epidemiology

Estimates on the prevalence of *Entamoeba* infection range from 1–40% of the population in Central and South America, Africa, and Asia, and from 0.2–10.8% in endemic areas of developed countries, such as, the USA (Dans & Martinez, 2005). In industrialized countries, amoebiasis occurs in sexually active homosexual men, immigrants, tourists who travel to areas of endemic infection, institutionalized persons, and human

immunodeficiency virus HIV-positive individuals. The best current estimate is that *E. histolytica* causes between 34 million to 50 million symptomatic infections (i.e. amoebic colitis or liver abscess) each year (Walsh, 1986). It is responsible for up to 100,000 deaths per year, mainly in Central and South America, Africa, and India, as well as for considerable morbidity manifested as invasive intestinal or extra-intestinal clinical features (WHO/PAHO/UNESCO, 1997). However, the true epidemiology of *E. histolytica* infection remains unknown, because much of the published epidemiological data fail to distinguish between *E. histolytica* and *E. dispar*. Fortunately, most of the individuals who were previously believed to have asymptomatic infection with *E. histolytica* are now known to be *E. dispar*, which has never been shown to cause invasive human disease (Diamond & Clark, 1993). Irrespective of high prevalence of invasive amoebiasis in male, asymptomatic infection with *E. histolytica* was found to be the same in both genders (Acuna-Soto *et al*., 2000). The occurrence of amoebic liver abscess is probably 5–50 times less common than that of intestinal amoebiasis in different geographic areas (Walsh, 1986).

In a survey of 50 communities covering the central Ethiopia, *E. histolytica* was reported in 45 of the communities with a prevalence rate ranging from 3% to 55% (Kloos & Tesfa Yohanes, 1993). In a recent study on the epidemiology of infections with intestinal parasites and HIV in Ethiopia, it was noted that *E. histolytica*/*E. dispar* was among the most common parasitic infection with a prevalence rate of 25% (Fontanet *et al.,* 2000). During the first species-specific diagnosis performed in central Ethiopia (Wonji), isoenzyme analysis showed that 2 of the 29 cultured *Entamoeba* trophozoites (6.9%) were zymodymes for *E. histolytica* zymodymes (Gatti *et al*., 1998). More recent, PCR based methods also showed that there is little or no true infection with *E. histolytica* (Kebede *et al*, 2003; 2004a; 2004b, Gebre-Tsadik *et al*., 2004).

1.8. Statement of the problem

Clinical reports based on routine diagnosis relying on observation of motile trophozoites and quadrinucleated cysts by microscopy give the impression that intestinal amoebiasis is rather common in Ethiopia. However, recent highly sensitive and specific tests based on

genetic differences revealed little or no true infection with *E. histolytica* in Ethiopia suggesting the inadequacy of microscopy as a method of diagnosis (Kebede et *al*., 2003; Gebre-Tsadik *et al*., 2004; Kebede *et al*., 2004a; 2004b). Retrospective studies in Tikur Anbessa hospital showed that there were only few cases of amoebic liver abscess in the last 20 years as compared to higher reports of intestinal amoebiasis supporting the idea that intestinal amoebiasis is overestimated in Ethiopia (Kebede *et al*., 2004c). Despite a great deal of scientific evidences available, the presence of *E. histolytica/E.dispar* as indicator of infection with *E. histolytica* in most of the health institutions in developing countries including ours is very common (Kebede *et al*.2003;2004a; 2004b; Leiva *et al*., 2006). Such widespread practice shows that people are being miss-treated with anti-amoebic drugs for the presence of no pathogen or different pathogen responsible for diarrhoea.

Clinical diagnosis of amoebiasis is difficult because of the nonspecific nature of the symptoms, which is easily confused with bacillary dysentery by *Shigella* spp, *Campylobacter jejuni, Yersinia enterocolitica, or enteroinvasive Escherchia coli* which are associated with grossly bloody or haeme-positive stools (Patel & DeRider, 1989; Reed, 1998). In view of high frequency of *E.dispar* in many areas, dysentery due to enteritis such as shigellosis or campylobacter will probably be misdiagnosed as amoebic colitis if microscopy is the sole diagnostic criteria (Stanley, 2003). Cases could be more severe in developing countries where both bacillary and amoebic infections are endemic in the absence of well-developed laboratories to differentiate between the two infections. Differential diagnosis must be employed in patients presenting with dysentery. There is also a trend of generalizing dysentery to intestinal amoebiasis even in the absence of trophozoites in the stool.

Therefore, the purpose of this study was to asses the extent of association of the commonly reported trophozoites *Entamoeba* spp. and the entero-pathogenic bacteria among diarrheal patients in Addis Ababa.

2. Objectives of the study

2.1 General Objective

To determine the extent of association of *Entamoeba* spp. trophozoites in diarrheal patients.

2.2 Specific objectives

1. To identify amoebic infection by microscopy among diarrheal patients.

2. To confirm microscopic positive *E. histolytica*/*E. dispar* trophozoites in diarrheal patients with species specific PCR tests.

3. To determine the common diarrhea causing enteric bacteria among diarrhea patients.

4. To examine the association of diarrhea with trophozoites of *Entamoeba* species and/or the common enteric bacteria.

5. To evaluate the appropriateness of fecal leukocytes in differential diagnosis of amoebiasis and shigellosis.

3. Materials & Methods

3.1. The Study population

The study subjects include 150 outpatients in Selam Health Center and Propride Clinic in Gulelie and Addis-Ketema sub-cities of Addis Ababa, respectively. The cases visited the respective health institution from December, 2005 to April, 2006 because of acute or chronic diarrhea. Diarrhea was defined as the passage of three or more loose or watery stools within 24 hours (Yousef *et al*., 2000). HIVserostatus of the patients was unknown except for the three HIV positive study participants who were being followed by the Addis Ketema Propride Clinic. History of patients including their age, sex, frequency and duration of diarrhea, clinical symptoms, and previous medical cares sought was recorded for each study participant by physicians at the respective health institutions.

3.2. Sample collection

About 3.5ml (3.5gm) fresh stool was collected from each study participants using sterile plastic cups having no preservative and immediately examined macroscopically for its consistency, appearance, and the presence of gross blood and mucus. Wet mount of each specimen using 0.9% normal saline was examined microscopically for the presence of leukocytes, red blood cells (RBC), *E. histolytica/E. dispar* and other protozoal and helminthic parasites at the respective health institutions. One milliliter of each sample was apportioned into a vial containing 3 ml of sodium acetate acetic formalin (SAF) for formol-ether concentration method to be done at EHNRI parasitology laboratory. Another 1ml of the same sample was apportioned in vial containing 3ml of absolute ethanol for DNA extraction. Four hundred micro-liters (µl) of the same specimen were apportioned in eppendorf tube containing 400µl of diluent for *E. histolytica* antigen ELISA. A swab of each sample was also preserved in a 3 ml transport medium, Carry-Blair medium (BBL), for isolation of enteric bacteria and was soon transported to EHNRI bacteriology laboratory.

3.3. Microscopic Detection of Parasite

A portion of each SAF preserved specimens was processed by formalin-ether concentration technique at EHNRI as described in Cheesbrough (1998). About one milliliter of each specimen was transferred into plastic vial after being filtered with cotton gauze. Then 8ml of 10% formalin and 3ml of diethyl-ether were added and centrifuged at 2000 rpm for 2 minutes. After decanting the supernatant the residue was transferred on to microscope slide. The slide was examined under the microscope at 100x and 400x magnifications for the presence of cysts and ova of parasites. Wet mount of the preserved samples was observed after mixing with 0.9% normal saline for the presence of vegetative stages of the parasites.

3.4. DNA extraction and PCR amplification

One milliliter of liquid or approximately (1g) of solid stool was suspended in 3 ml of absolute ethanol from each diarrheic patients involved in this study. The alcohol preserved samples were stored at 4 ^{0}c until DNA extraction. DNA was extracted from the stool samples using the QIAamp DNA mini kit (Catalog no. 51304; Qiagen inc., Mississauga, Ontario, Canada) according to the manufacturer's instruction (Verweij *et a*l., 2000). Approximately 250µl of the stool suspension in absolute ethanol was applied in a clean 1.5 ml eppendorf after the suspension being thoroughly mixed by vortexing. The ethanol was then removed after centrifuging the suspension at 10,000 rpm for 1 minute. The pellet was re-suspended in 750 µl 0. 9% NaCl (PBS), mixed, centrifuged at 10,000 rpm for 1 minute, and the PBS was removed using micropipette. After the washing step has been completed, 200µl of polyvinylpolypyrolidone (PVPP) was added and incubated at 100 ^{0}c in a heat block. It was soon short spinned and an equal volume of tissue lysing buffer (ATL) containing 20µl of Proteinase-K was added and thoroughly mixed by vortexing. The suspension was then incubated for 2hrs at 55 ^{0}c. After the incubated suspension being short spinned, 400 µl of AL buffer was added and thoroughly mixed by vortexing. The resulting suspension was then incubated at 70 ^{0}c for 10 minutes. It was then short-spinned and the supernatant was transferred into a clean eppendorf

containing 400 µl of absolute ethanol and mixed thoroughly. Numbered spin columns were placed a clean 2 ml collection tube and half of the suspension was poured into the column and then centrifuged at 10,000 rpm for 1 minute. The column was then placed in clean collection tube and the remaining suspension was poured and similarly centrifuged. The DNA stacked at the wall of the columns was subsequently washed by adding 500 µl of AW1 and AW2 buffers, and centrifuging at 10,00rpm for 1 minute at full speed for 3 minutes. After the washing step has been completed, the DNA was eluted into a 1.5 ml clean eppendorf by adding 200µl of AE buffer.

PCR amplification was carried out with the following specific forward and reverse primers: EV3-GGA CCC TCA TAC GTG AAA GTG and EV5-GTA AGA GGC TTGC ATG TTA CC. Primer EV3 is complementary to the tRNA Val gene and EV5 is in the tRNA Glu gene in the array [VME5]. The sequence and organization of the array can be found at the following website: http://homepages.lshtm.ac.uk/entamoeba/units/units.htm. The amplification condition was: primary denaturation (94º C, 3 minutes); 35 cycles with 94º C, 30 seconds (denaturation); 58º C, 30 seconds (annealing); 72 º C, 30 seconds (extension). Final extension was 72 º C for 7 minutes. The PCR product was visualized on 2 % agarose gel. The size of the PCR products for *E. histolytica* and *E. dispar* are expected to lie at 600bp and 800bp, respectively.

3.5. *E. histolytica/E. dispar* specific ELISA.

The fecal specimen was diluted by adding 400µl of diluent either to 400 µl liquid stool or to approximately 0.2g of solid stool. One drop of the conjugate (mouse monoclonal antibody specific for adhesion from *E. dispar* coupled to horseradish peroxidase) was added to each well. One drop of the positive control, 100 µl negative control (i.e. dilluent), and 100 µl of the diluted faeces were added to the respective well. The wells were then covered with plastic adhesive disks and incubated for 2hrs at room temperature. The assay wells were washed five times using diluted wash solution. Two drops of the substrate (Tetra methyl benzidine and peroxide) was added to all the test wells and re-incubated at room temperature for 10 minutes. Finally, the reaction was stopped by adding one drop of the stop solution (1M sulfuric acid). The resulting color

change (from blue to yellow) was measured at 450 nm on micro-plate ELISA reader .The specimen was considered as positive if their optical density (OD) reading is greater than 0.05 after deducing the OD value of the negative control from it.

3.6 .*E. histolytica* specific antigen ELISA

The *E. histolytica* specific antigen ELISA was performed using the test kit developed by the Tech lab (Blacksburg). Fecal specimens were prepared by diluting 400µl of liquid stool or approximately 0.2g of solid stool in 400µl of diluent available in the kit. One drop of the conjugate (mouse monoclonal antibody specific for adhesin from coupled with horseradish peroxidase) was added to each microtiter wells permanently coated with polyclonal antibody that binds adhesion of *E. histolytica*. One drop of the positive control, 100µl of the negative control (the diluent), and 200µl of the specimens were added to their respective wells.

The test wells were then covered with plastic adhesive sheet and incubated at room temperature for 2 hrs. The assay wells were washed five times using diluted wash solution. Two drops of the substrate (Tetra methyl benzidine and peroxide) was added to all the test wells and re-incubated at room temperature for 10 minutes. Finally, the reaction was stopped by adding one drop of the stop solution (1M sulfuric acid). The resulting colour change (from blue to yellow) was measured at 450 nm on micro-plate ELISA reader .The specimen was considered as positive if their optical density (OD) reading is greater than 0.05 after deducing the OD value of the negative control from it.

3.7. Isolation and characterization of enteric bacteria

A swab of stool samples collected from each study participants was inoculated onto MacConkey agar (MAC), Salmonella–Shigella agar (SSA), and Xylose Lysine Desoxycholate agar (XLD) as described by (WHO/CDC standard guide line). Portion of the specimens were also enriched in selinite F broth and sub-cultured later on SSA. All plates were incubated at 35^0C - 37^0C for 18-24 hours and examined for the presence of

lactose non-fermenting colonies and also lactose fermenting colonies for under-five children.

3.7.1. Isolation of *Salmonella* and *Shigella* spp.

Suspected *Salmonella* and *Shigellae* like colonies (non-lactose fermenters) were subsequently inoculated onto Kligler iron agar, motility, indloe, urea, manitole, and Simpson's citrate agar media. The isolates were then categorized as *Shigellae* like or *Salmonella* like based on the biochemical property of the two species. Confirmation and serotyping were done by slide agglutination using commercial group specific antisera (DIFCO).

3.7.2. Isolation of *Escherchia coli*

The lactose fermenter colonies from under five children were inoculated onto biochemical media mentioned above. Confirmation was done with polyclonal antibodies A and B for those isolates that fulfilled the characteristic of *E.coli*.

3.8. Antimicrobial susceptibility test

Anti-microbial susceptibility tests were performed for each isolate on Muller-Hinton agar using the agar disc diffusion method recommended by Bauer *et al*. (1966). The antimicrobial agents used include: ampicillin (A), cotrimoxazole (Q), chloroamphenicol (C), Tetracycline (T), norfloacin (NOR), amoxicillin (AML), gentamicin (G), and nalidixic acid (NA). The zone of inhibition was measured by caliper and decisions were made whether the isolate was sensitive or resistant.

3.9. Data Analysis

Statistical analyses were made using SPSS software version12. Association between different variables was analyzed by Pearsons's chi-square(X^2). The values were considered significant if the P-values were less than or equal to 0.05.

3.10. Ethical consideration

The ethical consideration for this study was cleared from the ethical committee of Department of Biology, Addis Ababa University. Written consent was sought from study participants and patients were treated for free if they were positive for all parasite or bacterial pathogens.

4. Results

4.1. Clinical history

Out of one hundred fifty cases of acute and chronic diarrheal patients suspected to have amoebiasis and/or shigellosis, 122 were from Selam Health Center and 28 were from Propride Clinic in Gullelie and Addis Ketema subcities of Addis Ababa, respectively. Their age ranged from 1 to 80, with an average age of 25.2 years. Most of the patients 132(88%) had visited health institution due to acute diarrhoea (diarrheal duration of less than two weeks), while the remaining 12% were due to chronic diarrhea (diarrheal duration of greater than two weeks). There were 66 males and 88 females with the male to female ratio of 1:1.3. Most of the patients had complaints for one or more of the following clinical symptoms: 127(84.7%) abdominal pain, 81(54.0%) tenesmus, 71(47.3%) history of watery diarrhea, 55(36.7%) history of bloody-mucoid diarrhea, 79(52.7%) history of low-grade fever, 63(42.0%) increased flatulence, and 58(38.7%) vomiting and nausea. Weight loss was a rare occurrence, observed only in 13.3 % of the cases. Almost all the patients reported that they were using tap water. Patients who took antibiotic or antiparasitic treatments for the last two weeks were excluded from the study.Though weight loss is a rare occurrence; it was prominent in patients with chronic diarrhea. None of the patients had clinical symptoms matching amoebic liver abscess as examined by physicians.

4.2 Macroscopic and microscopic examination of the stool samples

Macroscopic inspection of the stool samples showed that 96(64%) were liquid stool and 54(36%) were soft. Gross blood and/or mucus was observed in in the stool of 49(32.7%) of the diarrheic patients. By microscopic inspection, fecal leukocytes (white blood cells) and fecal erythrocytes (red blood cells) were observed in 76(50.7%) and 34(22.7%) of the stool samples inspected resprctivelly. Of the patients with clinical history of bloody and/or mucoid diarrhea, 49(89.1%) showed bloody diarrhea at the time of diagnosis, 42(76.4%) showed fecal leukocytes, and 22(40%) showed fecal erythrocytes (RBCs) in the stool. The association bloody mucoid diarrhea, fecal leukocyte and fecal erythrocyte

was highly significant (P<0.05). These stool findinds were also significantly associated with the clinical history of low-grade fever (P<0.05). However, these stool findings were significantly lower in patients with a clinical history of watery diarrhea in only sixteen of the seventy one (22.5%) of the patients with history of watery diarrhea presented bloody-mucoid stool at the time of diagnosis.

4.3 **Microscopic diagnosis of *E. histolytica /E.dispar* and other intestinal parasites**

Microscopic based investigation of the stool indicated that *E. histolytica /E.dispar* is the most common parasite among the study subjects. It was diagnosed in 40.7 % of the cases followed by *Blastocystis hominis* (24.7%), and *Giardia lambilia* (12.7%). All the cases of *E. histolytica/E. dispar* were in trophozoite stage, only one of the cases was diagnosed for the cyst stage. The detail is shown in (Table 2) below. The prevalence of intestinal helmiths was very low in the study subjects, only 3 (2%) of the diarrheic patients were positive for *Ascaris lumbricoides*, 2(1.3%) for *Hookworm* spp., 2(1.3%) *Strongyloid stercoralis* 2(1.3%) and *Hymenolepis nana* 2(1.3%).

Table 2. Prevalence of intestinal parasites among the 150 diarrheal patients diagnosed by direct microscopy and formol-ether concentration method from Selam Health Center and Pro-pride Clinic, Addis Ababa , December, 2005-April, 2006.

Detected parasite	Number of cases	Percent
E. histolytica/E. dispar	61	40.7
Blastocystis hominis	37	24.7
Giardia lamblia	19	12.7
Ascaris lumbricoides	3	2
Strongyloides stercoralis	2	1.3
Hookworm spp.	2	1.3
Hymenolepis nana	2	1.3
Trichuris trichiuria	2	1.3
Iodoamoeba butchili	1	0.7
Taenia spp	1	0.7

Finding of *E. histolytica*/*E. dipar* was specific to none of the clinical symptoms reported by the patients (P>0.05) (Table 3). Most of the patients reported a diarrheal duration of less than week, which is the characteristic of bacterial pathogens rather than amoebic infection. Though abdominal pain and tenesmus were among the most complaints reported by 84.7 % and 54 % of the cases respectively, no significant association was observed between these clinical symptoms and microscopic finding of *E. histolytica/E. dispar* (P>0.05). No association was also observed with the clinical history of watery or bloody diarrhea or with stool findings of pus cells or RBCs (P>0.05). RBC engulfing trophozoites which are the distinctive features of true infection with *E. histolytica* were not observed.

Table 3.Diagnosis of *E. histolytica/ E. dispar* by Microscopiy and the polymerase chain reaction (PCR) method in relation to the clinical history of diarrheal patients at Selam Health Center and Propride Clinic, Addis Ababa December, 2005-April, 2006.

Clinical history	Diarrheal cases (n=150)	*E. histolytica / E.dispar*(%) (n=61)	*E. dispar* PCR (%) (n=8)	*E. histolytica* PCR (%) (n=2)
Acute diarrhea	132	50(82)	8(13)	2(100)
Chronic diarrhea	18	11(18.0)	0	0
Abdominal pain	127	52(85.2)	7(87.5)	1(50)
Tenesmus	81	36(59.0)	7(87.5)	0
Clinical Hist. of BMD*	55	23(37.7)	3(37.5)	2(100)
Flatulence	63	28(45.9)	3(37.5)	0
Hist*.of low-grade fever	79	28(45.9)	2(25)	2(100)
Hist*. of watery diarrhea	71	29(47.5)	3(37.5)	0
Weight loss	20	5(8.2)	1(12.5)	1(50)
Vomiting and nausea	58	27(44.3)	5(62.5)	0

Hist*. History BMD* bloody mucoid diarrhea
Note: One patient may report more than one clinical symptom.

Finding of *Entamoeba* trophozoites with bacterial pathogens as well as with other intestinal parasite was not uncommon. As twenty-five (43.1%) of the *E. histolytica/ E. dispar* cases were diagnosed for bacterial pathogens, twenty (32.8%) for *B. hominis*, nine (14.8%) for *G. lamblia*, two (3.3%) for *Hookworm* spp. and *S. stercoralis*, and one (1.6%) for *T. Trichuira*, *H. nana* and *Escherichia coliB* (Table 4). None of the cases were significantly associated with the finding of *Entamoeba* trophozoites (P>0.05).

Table 4. Microscopic finding of trophozoites of *Entamoeba* spp. in the study participants co-infected with other parasites and diarrheagenic bacterial pathogens isolated by standard culture among 150 diarrheal patients diagnosed in Selam Health Center and Propride Clinic, Addis Ababa, December, 2005- April, 2006.

Other parasites and bacterial pathogens	*E. histolytica/E. dispar* Positive (%)	Negatve (%)	Total (%)
Blastocystis hominis	20(32.8)	17(19.1)	37(24.7)
Giardia lamblia	9((14.8)	10(11.)	19(12.7)
Strongyloides stercoralis	2(3.3)	0	2(1.3)
Hookworm spp.	2(3.3)	0	2(1.3)
Trichuris trichiuria	1(1.6)	1(1.6)	2(1.3)
Hymenolepis nana	1(1.6)	1(1.6)	2(1.3)
Ascaris lumbricoides	0(0)	2(2.2)	2(1.3)
Shigella spp.	25(43.1)	32(36)	57(38)
Escherichia coli B	1(1.6)	0	1(0.7)
Salmonella spp.	0	0	0
*Campylobacte*r spp.	0	0	0
Other categories*	0	29(19.3)	29(19.3)
Total	61(40.7)	89(59.3)	150

Note: Patients with *E. histolytica/E.dispar* could be coinfected with two or more pathogens.

* No pathogen detected.

4.4 Comparison of microscopy with PCR based method

Irrespective of the high prevalence of *E. histolytica/E. dispar* detected by microscopy, the species-specific PCR based method detected only eight cases of *E. dispar* and two cases of *E. histolytica*. The remaining 51 samples showed no amplification for both species. Sensitivity of microscopy to detect *E. histolytica/E. dispar* in this study was 100% whereas its specificity was 63.6%. The positive and the negative predictive value of microscopy to *E. histolytica/E. dispar* was 16.4%and 100% respectively (Table 5).

Table 5.Comparison of microscopic and species specific PCR based method for diagnosis of *E. histolytica/E. dispar* among 150 diarrheal patients diagnosed at Selam Health Center and Propride Clinic, Addis Ababa, December, 2005-April, 2006.

	PCR			
Microscopy	*E. histolytica*	*E. dispar*	Negative	Total
Positive	2	8	51	61
Negative	0	0	89	89
Total	2	8	140	150

4.5. *E. histolytica/E. dispar* specific atigen detection by ELISA

The *Entamoeba* specific ELISA detected 30 cases as *E. histolytica/E. dispar*; however only two of the cases were detected by species-specific PCR based method as *E. histolytica/E. dispar* (Table 6). Similarly only seven of them, were detected by microscopy as *E. histolytica/E. dispar* data not shown. The *E. histolytica/E. dispar* specific antigen detection ELISA was specific neither to microscopy nor to the species specific PCR based method. The sensitivity and specificity of *Entamoeba* specific ELISA to diagnose *E. histolytica/E. dispar* was 20% and 80% respectively, using the PCR based method as the gold standard. One of the two PCR positive samples for *E. histolytica* was also detected as *E. histolytica* by *E. histolytica* specific antigen detection ELISA.

Table 6. Comparison of *E. histolytica/E. dispar* specific antigen detection ELISA with the PCR based method among 150 diarrheal patients diagnosed at Selam Health Center and Propride Clinic, Addis Ababa December, 2005-April, 2006.

		E. histolytica/E. dispar PCR		
		Positive	Negative	Total
Entamoeba ELISA				
	Positive	2	28	30
	Negative	8	112	120
Total		10	140	150

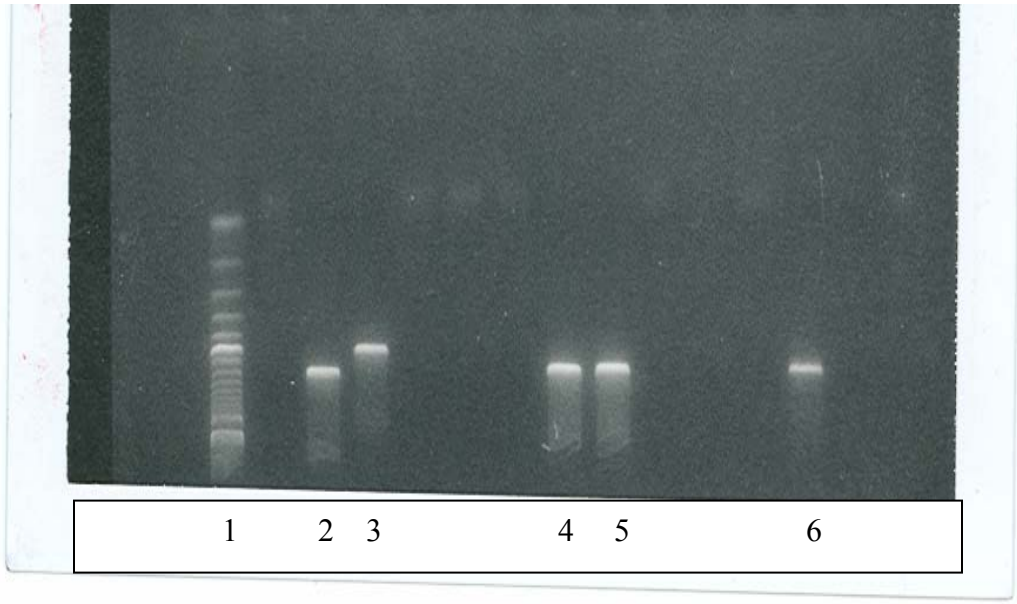

Figure 2. Agarose gel separation of PCR-amplified products of the short tandem repeating units of tRNA genes of *E. histolytica* and *E. dispar*: 1(100 base pair marker), 2 (*E. histolytica* HM1 positive control), 3(*E. dispar* SAW positive control), 5-6(*E. dispar*).

4.6. Enteric bacteria culture

Of the 150 samples cultured for enteric bacteria, the biochemical method identified 57(38%) as a member of *Shigellae*; however, the commercial available anti-sera serologically confirmed only 47 (82.5%) isolates. *Shigella flexneri* (group B) was the most prevalent sero-group accounting for 59.6% of the shigella isolates followed by *S. sonnei* (8.8%) and *S. boyedii* and *S. dysentrae* each accounting 7.0% (Table 7). The remaining 10 isolates were untypable by the anti-sera available. One of the 10 untypable shigella isolates showed biochemical feature typical to that of *S. dysentriae* based on the mannitol test. The rest exhibit biochemical features similar to that of *S. flexneri*. One *Escherichia coli* strain *B* was identified from diarrheal samples taken from under five children. *Salmonella* spp. and *Campylobacter* spp. were isolated from none of the stool samples tested.

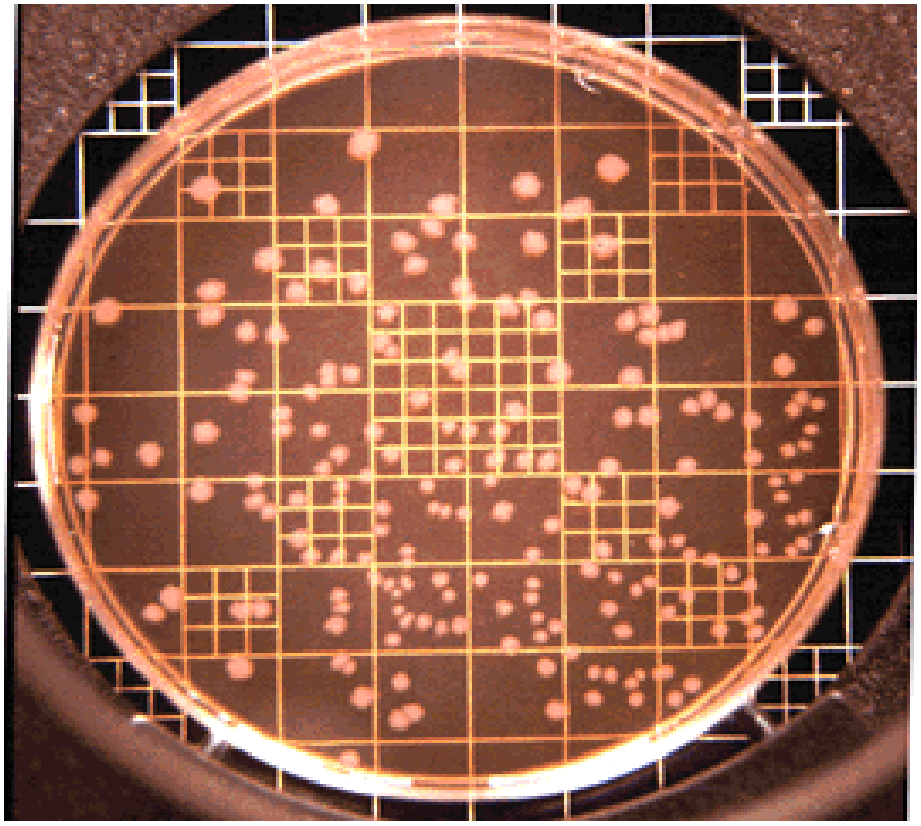

Figure 3.Appearance of *Shigellae* on MacConkey agar

Table 7.The sero-group prevalence of Shigella isolates among 150 diarrheal patients from Selam Health Center and Propride Clinic, Addis Ababa, December, 2005- April, 2006.

Type	Number of cases	% within *Shigellae*
Shigella dysenteriae	4	7
Shigella flexneri	34	59.6
Shigella boyedii	4	7
Shigella sonnei	5	8.8
Untypable *Shigella* spp.	10	17.6
Total	57	100

Shigellae was isolated from 34 /55 (61.8%) of the patients with clinical history of bloody-mucoid diarrhea, 44/79(55.7%) of patients with a clinical history of low-grade fever and 19/71(26.8%) of the patients with clinical history of watery diarrhea (Table 8). The isolation of *Shigella*e was significantly higher in patients with clinical history of bloody-mucoid diarrhea and low-grade fever ($P<0.05$). It was however, significantly lower in patients with clinical history of watery diarrhea ($P<0.05$). However, no significant association was observed with other clinical symptoms such as abdominal pain, tenesmus, flatulence, duration of diarhea (acute or persistent), and vomiting & nausea.

Regardless of the consistency of the stool, bacterial pathogens were observed in 31(63.3%) of the patients with bloody-mucoid diarrhea at the time of diagnosis. Bacterial pathogens were isolated in 50(65.8%) of the patients with microscopic fecal leukocytes and 23(67.6%) of the patients with microscopic fecal erythrocytes. Finding of gross blood and/ or mucus in the stool, microscopic finding of leukocytes and RBC were significantly associated with the isolation of *Shigella* spp. ($P<0.05$). The isolation was also lower in patients who submitted watery diarrhea at the time of diagnosis. Among the shigella serogroups, *Shigella flexneri* was significantly associated with clinical history of bloody diarrhea, low-grade fever, and stool findings of gross blood, leukocytes, and erythrocytes ($P<0.05$).

Clinical diagnosis of shigellosis was possible in 61.8% of the patients with clinical history of bloody-mucoid diarrhea and 55.7% of the patients with clinical history of low-grade fever. Similarly diagnosis of shigellosis was possible in 63.3% of the patients who sumited bloodymucoid stool at the time of diagnosis. The sensitivity and specificity of fecal leukocytes for the prediction of shigellosis was 86.2% and 71.7%, respectively. The positive and the negative predictive value of this method was 65.8% and 78.6%, respectively. However, the sensitivity of fecal erythrocytosis was lower (39.7%) but with relatively higher specificity (88.4%) and negative predictive value (76.4%).

Table 8. The association of clinical history and stool findings with the isolation of bacterial pathogens (*Shigella* spp.) among 150 diarrheal patients at Selam Health Center and Propride Clinic in Addis Ababa, December, 2005-April, 2006.

		Bacterial pathogens		Total	P-value
		Positive	negative		
History of BM* diarrhea	Positive	34	21	55	
	Negative	24	71	95	0.00
	Total	58	92	150	
History of low-grade fever	Positive	44	35	79	
	Negative	14	57	71	0.00
	Total	58	92	150	
History of watery diarrhea	Positive	19	52	71	0.005
	Negative	39	40	79	
	Total	58	92	150	
Bloody mucoid diarrhea	Positive	31	18	49	
	Negative	17	74	101	0.00
	Total	58	92	150	
Watery diarrhea	Positive	16	31	47	
	Negative	42	61	103	0.43
	Total	58	92	150	
Fecal leukocytes	Positive	50	26	76	
	Negative	8	66	74	0.00
	Total	58	92	150	
Fecal erythrocytes	Positive	23	11	34	
	Negative	35	81	116	0.00
	Total	58	92	150	

BM* = bloody mucoid

History of watery diarrhea= before hospitalization

Watery diarrhea = during sample collection

The isolation of *Shigella* spp. showed a prevalence rate of 17.6%, 33.3%, 41.9% and 57.1% among diarrheic patients in age range of 0-4years, 5-14 years, 15-59 years, 60 and above years, respectively (Fig.3). These prevalence rates showed a tendency to increase with age. However, no significant difference was observed among the different age groups (P>0.05). The prevalence of shigellosis was relatively higher in females of all age groups though no significant difference was observed.

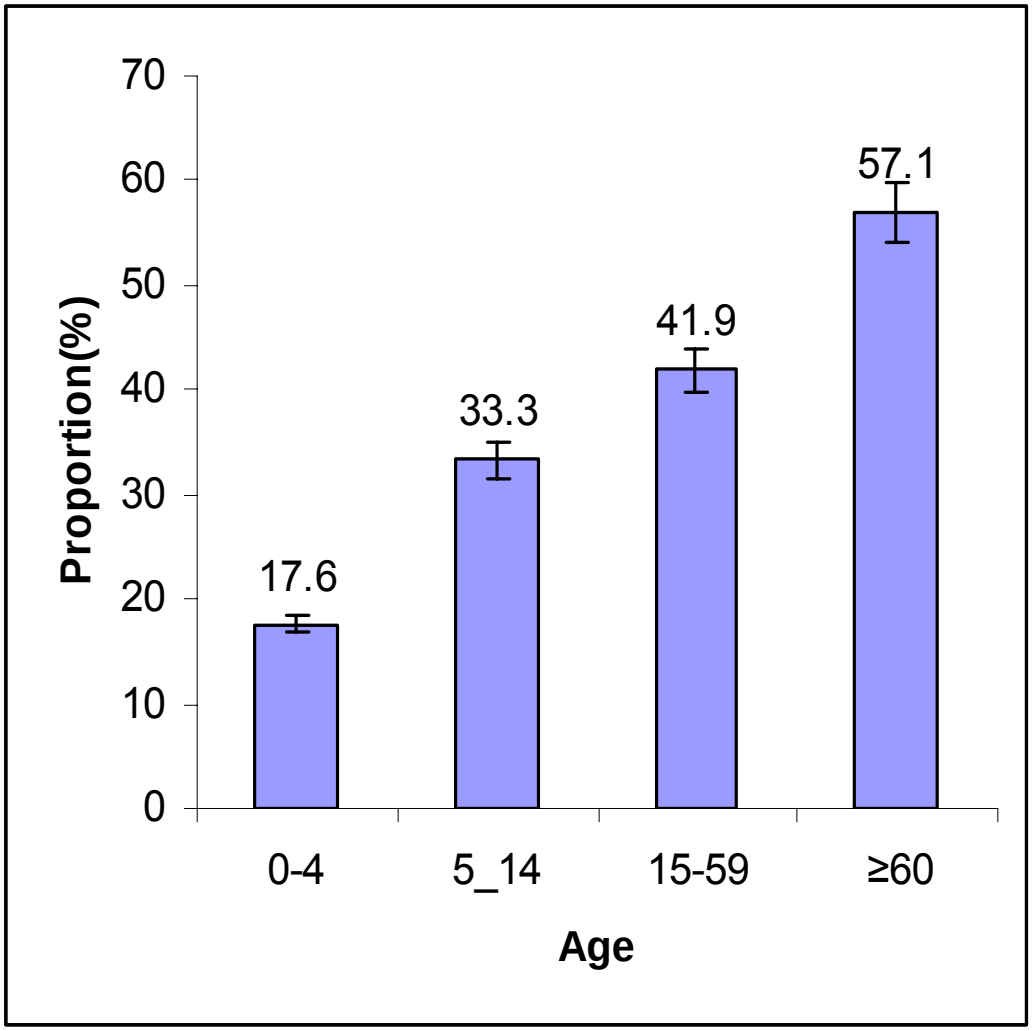

Figure 4. The distribution of *Shigella* spp.with regard to age among the 150 diarrheal patients by bacteriological method from Selam Health Center and Propride Clinic, Addis Ababa, December, 2005-April, 2006.

In attempt to compare the relative prevalence of *Shigella* spp. and trophozoites of *Entamoeba* spp., the isolation of *Shigella* spp. was significantly higher in patients with clinical history of abdominal pain, bloody mucoid diarrhea and low grade fever ($P<0.05$). On the contrary, the recovery of trophozoites of *Entamoeba* spp. was significantly higher in patients with clinical histories of watery diarrhea, tenesmus, and flatulence (Table 9).

Table 9.The relative frequency of *Shigellae* and *E. histolytica/E.dispar* trophozoites in relation to the clinical history of patients at Selam Health Center and Propride Clinic, Addis Ababa, December 2005-April, 2006.

Clinical history	*E. histolytica/E.dispar* (%) (n=36)	*Shigella* spp. (%) (n=33)	P-value
Acute diarrhea	29(80.5)	30(90.1)	0.00
Chronic diarrhea	7(19.4)	3(9.1)	0.13
Abdominal pain	31(86.1)	31(93.4)	0.00
History of watery diarrhea	20(55.6)	10((30.3)	0.03
History of B.M*. diarrhea	9(25)	20(60.6)	0.01
History low grade fever	11(30.6)	27(81.8)	0.01
Tenesmus	24(66.6)	17(51.5)	0.00
Flatulence	22(61.1)	14(42.4)	0.00
Weight loss	3(8.3)	8(24.2)	0.13

B.M*=bloody-mucoid

The isolation of *Shigella* spp. was significantly higher in patients with stool examination of occult blood, fecal leukocytes, and fecal erythrocytes as compared to recovery of trophozoites of *Entamoeba* spp. (Table 10). A significance difference in prevalence of *Shigella* spp. and finding of trophozoites of *Entamoeba* spp. was not observed in patients who sumited watery stool at the tie of diagnosis.

Table 10.The relative frequency of *Shigellae* and *E. histolytica/E.dispar* trophozoites in relation to the stool findings of patients at the time of diagnosis at Selam Health Center and Propride Clinic, Addis Ababa, December 2005-April, 2006.

Stool findings	*E. histolytica/E. dispar* (%) (n=36)	*Shigella* spp. (%) (n=33)	P-value
Watery diarrhea	7(19.4)	11(33.3)	0.11
Bloody mucoid diarrhea	7(19.4)	19(57.6)	0.02
Fecal erythrocyte	5(13.9)	17(51.5)	0.01
Fecal leukocytes	9(25.0)	30(90.1)	0.01

4.7. Antibiotic susceptibility test.

Most of the *Shigella* spp. (96.2%) showed antibiotic drug resistance to one or more of the antibiotics tested. The highest level of antibiotic resistance rate was shown against amoxicillin (81.5%) followed by tetracycline (79.2%), co-trimoxazol (71.7%), ampicillin (69.8%) and chloroamphenicol (41.5%) (Table 11). None of the strains tested showed resistance to norfloxacin, and about one quarter of the isolates that were tested for gentamicin and nalidixic acid showed no resistance to these two drugs. *Shigella flexneri* has shown the highest level of drug resistance to most of the drugs tested followed by *Shigella sonnei* and *Shigella dysenteirae*. The members of *Shigella boydii* (group C) showed the least resistance to most of the drugs tested. The resistance patterns of the untypable shigellae were almost similar to that of the *Shigella flexneri*.

Table 11. The antibiotic resistance pattern of *Shigella* spp. isolated from Selam Health Center and Prop ride Clinic in Addis Ababa by disc diffusion method (December, 2005-April 2006).

	Number of resistance strains (%)					
Antibiotics tested	*S. dysenteriae*	*S. flexneri*	*S. boyedii*	*S. sonnei*	Untypable	Total
Ampicillin	2(50)	28(82.4)	1(25)	1(20)	5(83)	37(69.8)
Cotrimoxazole	2(50)	26(76.5)	0(0)	5(100)	6(100)	38(71.7)
Chloroamphenicol	0(0)	20(58.8)	0(0)	0(0)	2(33.3)	22(41.50
Tetracycline	4(100)	26(76.5)	3(75)	5(100)	5(83)	42(79.2)
Norfloxacin	0	0	0	0	0	0
Amoxicillin	3(75)	31(91.2)	2(50)	1(20)	6(100)	40(85.1)
Total	4	34	4	5	6	54(100)

A multiple drug resistance pattern of 30.2%, 28.3%, 9.4%, and 15.1% to 5, 4, 3, and 2 antibiotics respectively were observed. *Shigella flexneri* showed the highest level of multiple drug resistance in that 41.2% of them showed multiple drug resistance to five drugs, namely, ampicillin, amoxicillin, tetracycline, chloroamphenicol, and co-trimoxazol. The remaining 32.4%, 5.9%, and 2.9% showed multiple antibiotic resistances to 4, 3, and 2 antibiotic drugs respectively.

5. Discussion

The present study indicated that *E. histolytica/E. dispar* is the most common parasite (40.7%) diagnosed by microscopy in diarrheal patients. The high prevalence of *E. histolytica/E. dispar* in diarrheal patients is comparable to what was reported in an earlier study in Ethiopia(Kebede *et al.,* 2003). The result was also in agreement with the existing prevalence data, which showed intestinal amoebiasis to be common intestinal infection in Ethiopia (Kloos & Tesfa-Yohanes, 1993). However, this prevalence data was based on microscopic identification of cysts and trophozites of *Entamoeba* spp. which cannot distinguish between the pathogenic *E. histolytica* and the commensal *E. dispar* (WHO/UNESCO, 1997). A prevalence rate of 24.7 % and 12.7 % for *B. hominis* and *G. lamblia* respectvely was noted in this study suggesting a high prevalence of intestinal protozoan parasites, which are mostly associated with poor hygienic conditions.

Unlike the microscopy based method, the species-specific PCR based method showed rather low prevalence of *E. histolytica/E. dispar* indicating that the trophozoites of non-pathogenic *Entamoeba* spp. other than *E. dispar* and/or fecal macrophages could have been wrongly identified as *E. histolytica/E. dispar*. These results indicate the poor reliability of microscopy for diagnosis of amoebiasis. Only 10 of (16.4%) of microscopic positive samples were confirmed to have *E. histolytica*/*E. dispar* by the species-specific PCR based method which amplified the short tandem repeating sequence of the tRNA. However, only two (20%) of the PCR confirmed cases were determined to be the pathogenic strain *E. histolytica* on species specific testing. One of the two E. histolytica was detected by anigen detection ELISA specific for both *E. histlytica* and *Entamoeba* spp. This finding is in agreement with the low concordance between microscopic species identification of *Entamoeba* trophozoites and PCR based determination as reported by earlier studies in Ethiopia (Kebede *et al*., 2003; Kebede *et al.,* 2004b) and elsewhere (Leiva *et al*., 2006), suggesting the miss-identification of fecal macrophages or trophozoites of non-pathogenic amoebae as *E. histolytica*. This could probably due to the morphological similarity trophozoites of *E. histolytica* with fecal macrophages or the trophozoites of other commonsal *Entamoeba* spp. This high probability of being missed

by PCR based method could probably be because all the *Entamoeba* cases were in the trophozoite stage. The high probability of trophozoites being missed by PCR than the cyst stage has been discussed by Kebede (2004a). As it has been indicated in an earlier study(Kebede *et al*., 2004b), it is not as simple as it is generally thought to identify RBC engulfing trophozoites, which is the distinguishing feature of the pathogenic *E. histolytica* in most clinical laboratory setup in Ethiopia .

In general, the low concordance between microscopy and PCR based method in this study could be explained in two ways: one, the sensitivity of PCR may be lower or microscopy is associated with over-diagnosis. The low sensitivity of PCR based method may have been caused because of poor DNA extraction method, DNA degradation in the stool samples, the presence of inhibitors in the stool, or the possible degradation of the dNTPs (Sandra *et al*., 2004, Leiva *et al*., 2006). The results of this study are consistent with previous studies that determined *E. histolytica* to be a rare finding among patients with diarrhea (Kebede *et al*., 2003; Leiva *et al*., 2006). This could be not only due to misdiagnosis of *E. dispar* but also as confusion of trophozoites of several other nonpathogenic intestinal amoebas or fecal macrophages as being *E. histolytica.* The possibility of such misdiagnosis where there is little or no true infection with invasive *E. histolytica* in Ethiopia has been well documented (Gathi *et al*., 1998; Kebede *et al*., 2003; Kebede *et al*., 2004a, b, c; Gebre-Tsadik *et al*., 2004).

Although no difference was observed in the prevalence of true infection with *E. histolytica*, the ratio of *E. histolytica* to *E. dispar* (1:4) was relatively higher in this study among the PCR confirmed cases of *E. histolytica/E. dispar*. This is contrasty to the finding of only 1/78(1.3%) PCR confirmed cases of *E. histolytica by* kebede *et al*. (2004b) in Ethiopia. Similarly only 2/29(6.9%) of the culture confirmed cases were the pathogenic zymodeme according to Gathi *et al*. (1998). This could probably due the sampling method or all the patients were symptomatic. This 1:4 ratio of *E. histolytica* to *E. dispar,* was however, comparable to what was reported by Leiva *et al*. (2006) for *E. histolytica* detection from other endemic areas.

The antigen detection ELISA specific to *E. histolytica/E. dispar* was much less sensitive than microscopy when verified with the PCR based method as the gold standard. This is in sharp contrast to the study conducted in Bangladesh, which showed the antigen detection ELISA to be as sensitive as the PCR based methods (Haque *et al*, 1998). This low sensitivity of antigen ELISA could be due to delay in the processing of samples, which actually need to be processed within a week. On the other hand, Gonin & Trudel (2003) had reported ELISA to be less sensitive than microscopy and PCR in differentiating *E. histolytica* from *E. dispar* in stool samples. Previous studies in Ethiopia have shown antigen detection by ELISA to be specific neither to microscopy nor to the PCR based method Kebede (2005). However, other studies from different countries have confirmed the simplicity, rapidity, and reliablty of antigen detection by ELISA (Furrows *et al*., 2004; Delialioglu *et al*., 2004; Redondo *et al*., 2006) indicating the possible influence of reagents and assay conditions in its reliability.

To address the possible cause of diarrhea in patients who were positive for trophozoites of *Entamoeba* spp., different diagnostic methods were performed for detection other intestinal parasites, which may cause diarrhea and other, associated syndromes. The high concurrent prevalence of protozoan parasites, most commonly *Blastocystis hominis* and *Giardia lamblia*, in patients with *Entamoeba* trophozoites suggests that some of the clinical symptoms could be attributed to these parasites. The absence of association between specific signs and symptoms and positive *Entamoeba* trophozoite infections makes the suggestion plausible. Most of the patients in this study presented with acute diarrhea instead of chronic, which by and large is the characteristic of bacillary dysentery rather than amoebic dysentery. This is because amoebic patients were shown to have a relatively longer period pre-hospital illnesses (20.8 days) compared to patients with shigellosis visiting hospital after an average diarrheal duration of 3.9 days (Speelman *et al*., 1987). These clinical findings reinforce the fact that there is little or no true infection with pathogenic *E. histolytica* in the study subjects.

The presence of trophozoites of *Entamoeba* spp. in the stool of patients with bloody diarrhea could not necessarily be an indicator of amoebiasis. Only one recent study conducted in Nigeria considered that *E. histolytica* is the most important cause of acute

diarrhea with such indicator in adults (Okeke *et al*., 2003). However, this study was entirely based on microscopic observation of cysts and trophozoites which failed to distinguish between *E. histolytica* and other non-pathogenic strains of *Entamoeba* more importantly the morphologically identical *E. dispar*. The study did not also consider the role of campylobacteriosis and yersiniosis as the possible causes of acute diarrhea. The same method also indicated *E. histolytica* to be the causative agent of childhood dysentery in Nigeria instead of *Shigella* (Ogunlesi *et al*., 2005). However, most of the study subjects (63.4%) in that study had received antibiotic treatment before hospitalization which might have masked the prevalence of *Shigellae* and consequently the possibility of co-infection by the two pathogens. Hence, it may be inappropriate to associate *Entamoeba* trophozoites detected in diarrheal patients the etiology of amoebiasis unless species-specific diagnosis is made. Rather it is better try to find other possible causes of diarrhea apart from the presence of *Entamoeb*a trophozoites (Gonzalez-Ruiz *et al*., 1994).

The other question to be raised here is that whether *E. histolytica* detected in the two stool samples is responsible for diarrhea as the two patients in whom *E. histolytica* detected were co-infected with *Shigellae*. Previous longitudinal studies showed that only 10% of individuals infected with *E. histolytica* in the South African study and in 3% of the Bangladesh study revealed symptoms of dysentery associated with amoebic infection during the 12-month observation period (Gathiram & Jackson, 1987; Haque *et al*., 1999). Since none of the study subjects in both studies developed an amoebic liver abscess, these studies suggested low probability of clinical amoebiasis due to infection with *E. histolytica*. Likewise in the present study, the absence of clinical symptoms compatible with amoebic liver abscess also supported the absence of clinical amoebiasis. A retrospective study at Tikur Anbesa Hospital by Kebede *et al*. (2004c) also showed the low prevalence of hepatic amoebiasis in Ethiopia regardless of the high reporting of intestinal amoebiasis. This is in sharp contrast to the commonly reported high prevalence of intestinal amoebiasis on the bases of detecting trophozoites or cysts of amoebae in the stool. Similar phemenon was observed if microscopy was to be used as the only criterion for diagnosis in the present study.

The result in this study indicated that diarrheic patients diagnosed for trophozoites of *Entamoeba* spp. are wrongly given anti-amoebic drugs without verification of the targete organism. This is in contradiction to the fact that the majority of the patients are most likely infected with a type of amoeba that does not cause symptoms. However, it is not unusual for most of the cases to get some sort of relief from the diarrhea after consuming anitamoebic drugs in the absence of the pathogenic *E. histolytica.* One possible explanation for this could be that the same antiamoebic drugs could also kill gram-negative anaerobic bacteria including members of the family *Entrobacteriacae* (Miller *et al*., 1999) that may have been responsible for the diarrhea.

The possible role of *Cryptosporidium, Microsporidia,* and *Isospora* as causative agents of diarrhea was not considered in this study. Yet, these parasites, too, are important diarrheagenic agents especially in HIV positive patients. However, since no HIV screening was done in the study their role in diarrhea was not considered. More over the study subjects present acute diarrhea rather than chronic diarrhea. The available data on the whole show in patients with bloody-mucoid diarrhea, enteric bacteria rather than *E. histolytica/E. dispar* trophozites were more frequently diagnosed among these patients and the occurrence of trophozoites of *Entamoeba* spp. was uniform in patients either with bloody or non-bloody diarrhea, reinforcing the idea that the trophozoites are of non-pathogenic.

The role of enteric bacterial pathogens as a cause of diarrhea was highlighted in 38% of the patients being *Shigella* spp. and 0.4% being *E. coliB.* The isolation rate of *Shigella* spp. in this study was comparable to an isolation rate of 33 % reported from the Ethio-Sweedish Children's Hospital (Kebede *et al*., 1999). The slight increase in the prevalence rate could be due to the fact that the patients in this study did not take antibiotic treatment before visiting the health institutions.

Although previous case control study conducted at Tikur Anbessa Hospital showed species of *Campylobacter* to be associated with diarrhea in Ethiopia (Asrat *et al*., 1999),

the 30 samples tested for campylobacteriosis did not show the growth characteristics of *Campylobacter* spp. Similarly, different *E. coli* strains carrying genes for different virulence factors were found to be prevalent in Ethiopia (Geyid *et al*., 1998), however, only one case of *Escherichia coli* was detected in this study. The isolation rate of diarrheagenic *Escherichia coli* could have been increased if molecular methods have been used. The absence of *Salmonella spp*. in this study is unusual as prevalence rates of 4.5% and 10.7% were reported in Addis Ababa by Ashenahi & Gedebou (1985) and Andualem & Geyid (2003) respectively.

Due to the fact that *Shigella* spp. can initiate infection at very low concentration, a false negative result by bacteriological method is expected. This low sensitivity of the culture method to detect *Shigella* spp. might also have underestimated the prevalence of shigellosis in diarrheal patients. According to a study conducted in Bangladesh the sensitivity of the culture technique was 72% and its specificity was 100%, when the PCR technique was considered as gold standard (Islam *et al*., 1998). Even lowered sensitivity of the culture method had been reported by different study (Dutta *et al*., 2001). These studies showed that false negative results due to failure of the culture to detect all the *Shigellae* are likely to happen. The lower sensitivity and specificity was attributed to the ability of *Shigella* spp. to cause clinical symptoms at a concentration too low to be detected by common bacteriological methods. The presence of non-*Shigellae* Gram-negative bacteria may also inhibit its growth on common bacteriological media. Regardless of the possible underestimation, the findings of the current study, however, indicate that shigellosis is the major problem as opposed to intestinal amoebaiasis as the cause of diarrhea.

Shigella flexneri was the most dominant species accounting for 59.6% of the *Shigellae* isolates followed by *S. sonei* (8.8%) and *S. boyedi* and *S. dysenteriae* each accounting for 7.0%. Similar studies conducted in Ethiopia showed the dominance of *Shigella flexneri* (Kebede *et al*., 1999; Roma *et* al., 2000). The dominant serogroup, *Shigella flexneri*, was responsible for most of the clinical symptoms and stool findings which characterized shigellosis in this study. The results of the present study were also supported by the fact

that *Shigella flexneri* is the dominant species in developing countries (Kotloff *et al*., 1999). Additional 10 isolates that showed biochemical reactions similar to *Shigella* spp. but that failed to agglutinate with commercially available anti-sera were also detected. Similar strains were diagnosed as untypable strains in other studies (Shigout) (Duta *et al*., 2001). One of the 10 untypable strains showed biochemical property of *S. dysenteriae* but the others were similar to that of *Shigella flexneri.* These strains are suggested to be subspecies of *S. flexneri* and *S. dysenteriae* (Dutta *et al*., 2001). The other possible explanation for this could be the poor quality of the antisera used or the closely related genus *Escherichia coli*, which may show similar biochemical properties.

The age group prevalence of *Shigella* spp. in diarrheic patients showed a slight increase with age which is contrary to the fact that Shigellosis is more prevalent in children under five years and infants (Kotloff *et al*., 1999). The low isolation rate of bacterial pathogens in children under five years could probably be due to lower number of cases under this age group or due to the absence of infants less than one year among the study participants.

The isolation of bacterial pathogens was significantly higher in patients who had a clinical history of bloody-mucoid diarrhea, low-grade fever and finding of gross blood in the stool. The importance of stool examination for gross blood is well established in diagnosis of dysentery (Stoll *et al*., 1983). The findings in the present study showed that careful recording of clinical histories together with stool findings could be important to distinguishing between amoebic and bacillary dysentery. Direct microscopical examination of stool specimens for the presence of WBC and RBC (Stoll *et al*., 1983; Speelman *et al*., 1987; Khan *et al*., 2006) and stool methylene blue stain for fecal polymorphonuclear leukocytes (DeWitt *et al*., 1985) was effective presumptive diagnostic test for invasive bacterial diarrhea. In this study, clinical histories of bloody-mucoid diarrhea, low-grade fever in association with fecal leukocytosis and microscopic observation of red blood cells in the stool of patients were good markers of shigellosis.

In this study, a good association was observed between isolation of bacterial pathogens and the clinical histories of bloody mucoid diarrhea, low grade fever and the stool findings for gross blood, WBC, and RBC. Hence, the result apparently suggests the importance of clinical dignosis combined with careful stool examination to be cheeper alternative for diagnosis of shigellosis in developing countries like Ethiopia where there are no bacteriological laboratories at the health center level.

High rate of antibiotic resistance was observed to most commonly used drugs like ampicillin & cotri-moxazol. Similar pattenrn of antibiotic resitance was observed by previous studies in Ethiopia (Kebede *et al*., 1999; Roma *et al*., 2000) suggesting the circulation of drug resistance strains. The emergency of multidrug resistance against the most common shigella strain in Ethiopia, *Shigella flexneri,* may indicate a serious public health problem. The highest level of multiple drug resistance against the most prevalent shigellae (Shigella flexneri) needs to reconsider treatment regimens not to include drugs like ampicillin, chloroamphenicl, cotrimoxazole etc.

6. Conclusions

1. The result in the present study shows the rare occurrence of true infection with pathogenic *E. histolytica* and the inadequacy of microscopy for diagnosis of amoebiasis.
2. The present study indicated not only overdiagnosis of intestinal amoebiasis due to the existence of morphologically identical *E. dispar* but also due to the presence of fecal macrophages and/or commensal *Entamoeba* spp. trophozoites other than *E. dispar*.
3. The laboratory settings in Ethiopia have failed to distinguish between RBC engulfing trophozoites and those who do not.
4. Microscopic recovery of trophozoites of *Entamoeba* species was specific to none of the clinical symptoms and stool findings.
5. The high rate of co-infection observed in patients with diarrhea causing parasites and enteric bacterial pathogens indicate that the trophozoites of *Entamoeba* species are incorrectly associated with diarrhea as a common practice simply for the presence of non-pathogenic amoeba.
6. Higher number of trophozoitess *of Entamoeba* species in stool specimens could not be necessarily associated with any clinical symptom but to a diarrhea of other etiologic agents such as shigellosis or giardiasis.

7. Recommendations

1. There is a need to improve the quality of microscopy and proper training of laboratory technicians with the appreciation of two distinct but morphologically identical species.
2. In view of its simplicity to perform and its low cost, the antigen ELISA needs to be evaluated using different distinct isolates from different endemic areas of Ethiopia.
3. Patients in whom trophozoites of *Entamoeba* spp. are diagnosed should not be treated unless species-specific diagnosis for the pathogenic *E. histolytica* is made or RBC engulfing trophozoites are observed microscopically. Rather, here is a need to look for other causes of diarrhea even in the presence of trophozoites of *Entamoeba spp*. or cysts.
4. There is a need to develop a rapid and easy method to distinguish between amoebic and bacillary dysentery that could be used at health center levels. Clinical history of low grade fever, bloody mucoid stool, and the microscopic finding of leukocytes and erythrocytes for differential diagnosis of bacillary and amoebic dysentery should be considered where there are no bacteriological laboratories at the health center level.
5. The contribution of enteric coccidians, like *Cryptosporidium parvum*, *Isospora belli,* and *Microsporidia,* must also be assessed in establishing the etiology of diarrhea in Ethiopia.
6. The higher isolation rate of diarrheagenic bacterial and parasitic pathogens indicates the need to improve personal & environmental hygiene.
7. Molecular methods to determine the real prevalence of diarrheagenic bacteria such as *Escherichia coli* and *Shigllae* need to be applied and the reliability of stool examination for WBC and RBC for diagnosis of shigellosis needs to be evaluated in different settings

8. References

Abd-Alla, M. D., Jackson, T. F. H. G., Gathiram, V., El-Hawey, A. M., & Ravdin, J. I. (1993). Differentiation of pathogenic *Entamoeba histolytica* infections from nonpathogenic infections by detection of galactose-inhibitable adherence protein antigen in sera and feces. *J. Clin. Microbiol*. **31**:2845–2850.

Abd-Alla, M. D., Jackson, T.G., and Ravdin, J. I. (1998). Serum IgM antibody responses to the galactose-inhibitable adherence lectin of *Entamoeba histolytica*. *Am. J. Trop. Med. Hyg.* **59**:431-434.

Ackers, J.P. (2002). The diagnostic implications of the separation of *Entamoeba histolytica* and *Entamoeba dispar. J.Biosc.***27** (*Suppl. 3*): 573–578.

Acuna-soto, R., Samuelson, J., DeGirolami, P., Zarate, L., Millan- Elcaso, F., Schoolnick, G. (1993) Application of polymerase chain reaction to the Epidemiology of *Entamoeba histolytica*. *Am.J.Trop.Med. Hyg*. **48**(1): 58-70.

Acuna-Soto, R., Maguire, J.H., & Wirth, D.F. (2000). Gender distribution in asymptomatic and invasive amoebiasis.*Am.J.Gastroenterol***.95**: *Abstract*.pubmed

Aguire, A., Warhurst, D.C., Guhl, F., & Frame, I.A. (1995). Polymerase chain reaction-solution hybridization enzyme linked immuno assay (PCR- SHELA) for the differential diagnosis of pathogenic and non-pathogenic *E. histolytica*. *Trans R Soc Trop Med Hyg.***72**: 187-188.

Ali, I.K.M., Zaki, M., & Clark, C.G. (2005).Use of PCR amplification of tRNA gene-linked short tandom repeats for genotyping *Entamoeba histolytica*. *J.Clin. Microbiol.* **43**: 5842-5847.

Allonson-Iones, E., Mindel, A., Sargeaunt, P.G., & Katz, D. *(*1988). Outcome of untreated infection with *Entamoeba histolytica* with and without HIV antibody. *BMJ*.**297**:654-657.

Andualem, B. & Geyid, A. (2003). The prevalence of Yersinia enterocolitica isolates in comparison to those of the commonly encountered enteropathogens causing diarrhoea among Ethiopian patients in Addis Ababa. *Eth. Med. J*.**41** (3):257-66.

Ashenahi, M. & Gedebou, M. (1985). Salmonella and Shigella in adult diarrhoea in Addis Ababa--prevalence and antibiograms. *Trans R Soc Trop Med Hyg.* **79**(5):719-21.

Asrat, D., Hathaway, A., &Ekwall, E. (1999). Studies on enteric campylobacteriosis at Tikur Anbessa Hospital, Addis Ababa, Ethiopia. *Eth. Med. J*.**37**:71-84.

Bauer, A.W., Kirby, W.M., Sherris, J.C., Turck, M. (1966) Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin .Pathol*. **45**:493–496.

Bhattacharya, S., Som, I., & Bhattacharya, A. (1998). The ribosomal DNA plasmids of *Entamoba. Parasitol Today*. **14**:182-185.

Cheesbrough, M. (1998). *District laboratory practice in tropical countries*, 2nd (Ed) Vol (1), Cambridge University Press, United Kingdom. P.194-198.

Clark, C.G. (1998). Amoebiasis: *Entamoeba dispar*, an organism reborn. *Trans. Trop. Med. Hyg*. **92**:361-363.

Clark, C.G., Zaki, M. & Ali, I.K.M (2002). Genetic diversity in *Entamoeba histolytica*. *J.Biosc*.**27**: 603-6-07.

Dans, L & Martinez, E. (2005).Amoebic dysentery. *Clin Evid*.**14**:1–7.

Delialioglu, N., Aslan, G., Sozen, M., Babur, G., Kanik, A., & Emekdas, G. (2004). Detection of *Entamoeba histolytica/Entamoeba dispar* in stool specimens by using enzyme-linked immunosorbent assay. *Mem. In. Oswaldo. Cruz*. **99**(7): 769-772.

DeWitt , T.G., Humphrey, K.F., & McCarthy, P. (1985). Clinical predictors of acute bacterial diarrhea in young children. *Pediatrics*. **76**:551–6.

Diamond, L.S. & Clark, C.G. (1993). A redescription of *Entamoeba histolytica* "Shaudinn1903 (emeded walker1911)" separating from *Entamoeba dispar* (Brumpt, 1925). *J.Eukaryo . Microbiol*.**40**: 340-344.

Duta, S., Chaterjee, A., Duta, P., Rajendran, K., Roy, S., Pramanik, K.C., Bhattacharya, S.K.(2001). Sensitivity and performance characteristic of a direct PCR with stool samples in comparison to conventional techniques for diagnosis of *Shigella* and enteroinvasive *Escherichia coli* infections in children with acute diarrhea in Calcata, India. *Diagn. Microbiol*.**50**:667-674.

Eldson-dew, R. (1968). The epidemiology of amoebiasis. *Adv. Parasitol*. **1**:1-60.

Entamoeba histolytica: Whole Genome Shotgun (2005). (http://www.sanger.ac.uk/).

Fontanet, A.L., Shalu,T., deWit, T.R., Messele, T., Woldemichael, T., Yeneneh, H.,& Coutinho, R.H.(2000). Epidemiology of infection with intestinal parasites and human immunodeficiency virus (HIV) among sugar-estate residents in Ethiopia. *Ann.Trop.Med. Parasitol*.**94**: 269-278.

Freitas, M.A.R., Vianna, E.N., Martins, A.S., Silva, E.S., & Pesquero, J.L. (2004). A single step duplex PCR to distinguish *Entamoeba histolytica* from *Entamoeba dispar*. *Parasitol*. 128: 625-628.

Furrows, S. J., Moody, A. H. & Chiodini, P. L. (2004). Comparison of PCR and antigen detection methods for diagnosis of *Entamoeba histolytica* infection. *J. Clin.Path.* 4:1264-1266

Garcia, L. S., & Bruckner, D. A. (1997). *Diagnostic medical parasitology,* 3rd ed. ASM Press, Washington, D.C.

Gathiram, V. & Jackson, T.F. (1987). A longitudinal study of asymptomatic carriers of pathogenic zymodemes of *Entamoeba histolytica*. *S Afr Med J.* **72**(10):669-72.

Gatti, S., Mahadi, R., Bruno, A., Cevini, C., & Scagalia, M. (1998). A survey of amoebic infection in the Wonji area of central Ethiopia. *Ann.Trop.Med. Parasitol*.**92**: 173-179.

Gebre-Tsadik, A., Kebede, A, Mekonnen, M., & Tadesse, G. (2004). Differentiation of two morphologically identical species of *Entamoeba*. *Eth. J. Hlth. Dev.***18** (2):121-125.

Geyid, A., Olsvik, O., & Ljungh, A. (1998). Virulence property of *Escherichia coli* from Ethiopian patients with acute and persistent diarrhea. *Eth. Med.J.***36**:123-138.

Gonzalez-Ruiz, A., Haque, R., Aguirre, A., Gaston, G., Hall, A., Ghul, F., Ruiz-Palacios, G., Miles, M. A., & Warhurst, D. C. (1994). Value of microscopy in the diagnosis of dysentery associated with invasive *Entamoeba histolytica*. *J. Clin. Pathol*. **47**:236-239.

Gonin, P., & Trude, L. (2003). Detection and differentiation of *E. histolytica* and *E.dispar* isolates in clinical samples by PCR and enzyme linked immunosorbent assay. *J. Clin. Microbiol.***41**: 237-241.

Guerrant, R.J. (1986). Amoebiasis: Current status and research question. *Rev. Infec.Di*.**8**: 218-227.

Haque, R., Neville, L.M. & Petri, W.Ajr. (1995). Rapid diagnosis of *Entamoeba* infection by using *E. dispar* and *E. histolytica* stool antigen detection Kits. *J. Clin. Microbiol*. **33** (1): 2558-2561.

Haque, R., Ali, I.K.M., Akther, S. & Petri, W.A. Jr. (1998). Comparison of PCR, isoenzyme analysis and antigen detection for diagnosis of *E. histolytica* infection. *J.clin. Micro. Biol*. **36**:449-452.

Haque, R., Ali, I. K. M. & Petri, W. A. Jr. (1999). Prevalence and immune response to *Entamoeba histolytica* infection in preschool children in Bangladesh. *Am. J. Trop. Med. Hyg*. **60**:1031-1034.

Haque, R. Huston, C.D., Huges, E. & Petri, W.A. (2003). Current concepts: Amoebiasis. *N.E.J.M*. 348:1565-1573.

Health and wellness (2007). Methylene Blue Stain, Stool. (http: //www. enh org./ healthandwellnes/encyclopeda/).

Huston, C.D., Haque, A., & Petri, W.A., Jr. (1999). Molecular diagnosis of *Entamoeba histolytica* infection. *Rev Molec. Med*. **1**:1-11

Islam, M.S., Hassan, M.K., Rahma, M.M., Fuchs, G., Mahalanabis, D., Baqui, A.H., & Albert, M.J. (1998). Detection of *Shigellae* from stools of dysentery patients by culture and polymerase chain reaction techniques. *J. Diarh. Dis*.**16**: 248-51

Jacobson, J.A. (1979). Shigellosis in adults: Diagnostic difficulties and delays. *West.J.Med.* **131**:349-351.

Juniper, K.Jr. (1984). Amoebiasis. *Phil.J.Infect. Dis*.**13**:49-64.

Kebede, A., Verweij, J., Dorigo-Zetsma, W., Sanders, E., Messele, T., Leshour, L.V., Petros, B., & Polderman, T. (2003). Over diagnosis of amoebiasis in the absence of *Entamoeba histolytica* among patients presenting diarrhoea, Wonji and Akaki, Ethiopia. *Trans. R. Soc. Trop. Med. Hyg*.**97:** 305-307.

Kebede, A., Verweij, J.J., Endeshaw, T., Messele, T., Tasew, G., & Petros, B., (2004a). The use of real-time PCR to identify *E. histolytica* and *E. dispar* infections in prisoners and primary-school children in Ethiopia. *Ann. Trop. Med. Parasitol*. **98**:43–48.

Kebede, A., Verweij, J.J., Petros, B., Polderman, A.M. (2004b). Short communication: Misleading microscopy in amoebiasis. *Trop. Med. Intern. Heal*. *Sup.***9**:651-52.

Kebede, A., Kassa, E., Ashenafi, S., Woldemicahel, T., Polderman, A.M., & Petros, B. (2004c). Amoebic liver abscess: a 20-year retrospective study at Tikur Anbessa Hospital, Ethiopia. *Eth. J. Hlth. Dev*.**18**:199-202.

Kebede, A. (2005). *Amoebiasis in Ethiopia: problems in diagnosis and determination of prevalence of infection* (PhD dissertation). Birhan and Selam Printing Enterprise, Addis Ababa.

Kebede, S., Geyid, A., Lulseged, S., & Mammo, K. (1999). Clinical profiles of and drug resistance pattern of the *Shigella* isolated from children in Addis Ababa. *Eth. Med. J.* 7:19-29.

Khan ,A.I., Huq , S., Malek ,M.A., Hossain ,M.I., Talukder ,K.A., Faruque , A. S. G., & Salam, M.A. (2006) Analysis of fecal leukocytes and erythrocytes in *Shigella* infections in urban Bangladesh. *J. Med. Microbiol*. **55**:1257-1263.

Khotaii, G., Hadipoor, Z., & Hadippor, F. (2003). Amoebic liver abscess in Iranian children. *Act.Med.Irania*.**41** (1). 33-36.

Kloos, H. & Tesfa-Yohanes, T. (1993). *Intestinal parasitism. In: the Ecology of health and disease in Ethiopia*.H.a.Z.A.Kloss, editor.Westviewpress, Oxford. P.223-235.

Kotlof, K.L., Winickoff, J.P., & Ivanoff, B. (1999). Global burden of *Shigella* infections: implications for vaccine development and implementation of control strategies. *Bull. W.H.O*.**77**: 651-666.

Leiva, B., Lobbed, M., Winecka-Krusnel, J., Altamirano, I., Tellez, A. & Linder, E. (2006). Over diagnosis of *Entamoeba histolyitca* and *Entamoebadispar* in Nicaragua: A microscopic, Triag parasitic pannel and PCR study. *Arc. Med. Res* **7**:529-534.

Lucas, R. & Upcroft, J.A. (2001). Clinical significance of the redefinition of the agent of amoebiasis. *Rev Latinoam Microbiol***. 43** (4): 183-187.

Martinez-Palomo, A., & Espinosa-Cantellano, M. (998). *Intestinal amoebae*. In: Cox EG, Kreier JP, Wakeline D, eds. Topley and Wilson's Microbiolology and microbial infections. Vol. 5, (9^{th} Ed.). P.157-177.

Mathur, S., Geholt, R.S., Mohta, A., & Bhargava, N. (2002). Clinical profiles of amoebic liver abscess. *J.I.A.C.M*. **3**:367-73.

Miller, N., Ende, J.V.D., Brink, A., & Botha, F. (1999).*Antibiotic guidelines*. 2nd(Ed) Ampath Trust. pp.18 http://www.ampath.co.za/AntiBiotGuide/main.htm.

Mirelman, D., Bracha, R., Wexler, A., & Chayen, A. (1986). Changes in isoenzyme patterns of a cloned culture of nonpathogenic *Entamoeba histolytica* during axenization. *Infect. Immun*. **54**:827-832

Mirelman, D., Nuchamowitz, Y., & Stolarsky, T. (1997). Comparison of use of enzyme-linked immunosorbent assay-based Kits and PCR amplification of RNA Genes for simultaneous detection of *E. histolytica* and *E. dispar J.Clin. Microbiol*.**35:** 2405-O7.

Ogunlesi, T.A., Okeniyi, J.A.O., Oyedeji, O.A., Oseni, S.B.A., Oyelami, O.A. & Njokanma, O.F. (2005). Childhood dysentery in Ilesa, Nigeria: The unusual role Of *Entamoeba histolytica. Internet J. Trop. Med.* **2**(2).
http://www.ispub.com/ostia/index.php?xmlFilePath=journals/ijtm/vol2n2/entamoeba.xm

Okeke, I. N., O. Ojo, A. Lamikanra, & Kaper**,** J. B. (2003). Etiology of acute diarrhea in adults in Southwestern Nigeria. *J. Clin. Microbiol*. **41:**4525-4530.

Patel, A.S. & DeRidder, P.H. (1989). Amoebic colitis masquerading as inflammatory bowel disease: the role of serology in its diagnosis. *J. Clin. Gasteroenterol.* **11**:407-410.

Paul, J., Bhattacharya, A., & Bhattacharya, S. (2002). Close sequence identity between ribosomal DNA episomes of the nonpathogenic *Entamoeba dispar* and pathogenic *Entamoeba histolytica. J. Biosci*. **27**(*Suppl. 3*): 619–627

Petri, W. A., Jackson, T. F. H. G Gatheram, V., Kress, K., & L. D. Saffer. (1990a). Pathogenic and nonpathogenic strains of *Entamoeba histolytica* can be differentiated by monoclonal antibodies to galactose-specific adherence lectin. *Infect. Immun*. **28**:1802-1806.

Petri W A Jr, Snodgrass T L, Jackson T F, Gathiram V, Simjee A E, Chadee K, &Chapman, M D. (1990b) Monoclonal antibodies directed against the galactose-binding lectin of *Entamoeba histolytica* enhance adherence. *J. Immunol*.**144**: 4803–4809.

Petri, W.A. (1996). Recent advances in amoebiasis. *Crit.rev.Clin.Lab.sci*.33:1-37.

Petri, W.A. & Singh, U. (1999). Diagnosis and management amoebiasis. *Clin.Infect. Dis.* **29***:* 117-1125.

Pillai, D. R., J. S. Keystone, D. C. Sheppard, J. D. MacLean, D. W. MacPherson, & Kain. K. C. (1999). *Entamoeba histolytica* and *Entamoeba dispar*: epidemiology and comparison of diagnostic methods in a setting of nonendemicity. *Clin. Infect. Dis*. **29**:1315-1318.

Ravdin, J.I., Abd-Alla, M.D., Welles, S.L., Reddy, S., & Jackson T.H.F.G. (2003). Intestinal antilectin immunoglobulin-A antibody response and immunity to *Entamoeba dispar* infection following Cure of amoebic liver abscess. *Infect Immu.***71**: 6899–6905.

Redondo, B.R., MéndeZ, L.G.M., & Baer, G. (2006). *E. histolytica* and *E.dispar:* Differentiation by enzyme-linked immunosorbet assay (ELISA) and its clinical correlation in pediatric patients. *Parasitol Latin.***61:**37-42.

Reed, S.L., Eckman, G.L., Kangof, M., & Mckerrow, J.H. (1996). *Molecular and cellular biology of invasion by E. histolytica*. In: *Parasitology of the 21st centuryCAB*, international, Turkey.

Reed, S. L. (1998). *Entamoeba histolytica* and other protozoan parasites. In: *Infectious disease* (2nd Ed), W.B, Saunders Company, Philadelphia. PP.2393-2399.

Roma, B., Worku, S., T/Mariam, S., & Langeland, N. (2000). Antimicrobial susceptibility pattern of Shigella isolates in Awassa, *Ethiop. J. Health Dev* **14**(2): 149- 154.

Roy, S. Kobir, M., Mondal, D., Ali, I. K. M., Petri, W.A., Haque, R. (2005). Real Time PCR Assay for Diagnosis of *Entamoeba histolytica* infection. *J. Clin. Microbiol*. **43**(25): 2168–2172.

Sandra M. B. P., Rosa M. C., Ivanise S. A., Joa˜ o I. I., Marcos A. M.Jr., Maria R. M. C., and Luiz B. C.A. JR. (2004). Determination of the prevalence of *Entamoeba histolytica* and *E. dispar* in the Pernambuco state of Northeastern Brazil by a polymerase chain reaction. *Am. J. Trop. Med. Hyg.* **70**(2):221–22

Sansonetti, P., & Phalipon A. (1996) Shigellosis: From molecular pathogenesis of infection to protective immunity and vaccine development. *Res. Immunol.* **147**:595–602.

Sargeaunt, P.G. & Williams, J.E. (1978). The differentiation of invasive and non-invasive *Entamoeba histolytica* by isoenzyme electrophoresis. *Trans. Roy. Soc. Trop. Med. Hyg*.**72**: 519-521.

Salvo, K.L., Baron, J.E., Tompkins, L.S., Passaro, D.J. (2000).Fecal leukocyte stain has diagnostic value for outpatients but not inpatients. *J Clin Microbiol*. **39**(1): 266–269.

Sharma, M.P., & Ahuja, V. (2003). Amoebic liver abscess. *J.I.A.C.M*. **4**: 107-11.

Smith, A.J. & Barrett (2000). The amoeba. In: *Foundation of parasitology. Schmitt and Roberts* (Ed), McGraw-Hill, pp.101-115.

Speelman, P., McGlaughlin, R., Kabir, I., & Butler, T. (1987). Differential clinical features and stool findings in shigellosis and amoebic dysentery. *Trans. Roy. Soc. Trop. Med. Hyg*.**81**: 549-551.

Stanley, S. L. (2003). Amoebiasis. *Lancet.* **361**:1025-1034.

Stoll, R.J., Glass, R.I., Banu, Huq, M.I., Khan, M.U. & Ahmed, M. (1983). Value of stool examination in patients with diarrhea. *B.M.J.* **286**:2037-2040.

Tachibana, H., Kobayashi, S., Kaneda, Y., Takeuchi, T, & Fujiwara, T. (1997). Preparation of a monoclonal antibody specific for *Entamoeba dispar* and its ability to distinguish *E. dispar* from *E. histolytica*. *Clin. Diag. Lab.Immunol.* **4**:409–414.

Tachibana, H., Cheng, X., Watanabe, K., TakekoshI, M., Maeda, F., Aotsuka, S., Kaneda, Y., Takeuchi, S., & Ihara, S. (1999). Preparation of recombinant human monoclonal antibody Fab fragments specific for *Entamoeba histolytica. Clin.Diagn.Lab.Immunol*.**6**: 383–387.

Tannich, E., Horstman, R. D., knobloch, J., & Arnold, H.H. (1989). Genomic DNA differences between pathogenic and nonpathogenic *Entamoeba histolytica*. *Proc. Nati. Acad. Sci. USA*.**86**: 5118-5122.

Tanyuksel, M., & Petri, W. A. (2003). Laboratory diagnosis of amoebiasis. *Clin.Microbiol.Rev*.16: 713-729.

Verweij, J.J., Blotkamp, J. Brienen, E. A., Aguirre, A., & Polderman, A.M. (2000). Detection and Differentiation of *Entamoeba histolytica* and *Entamoeba dispar* cysts using polymerase chain reaction on DNA isolated from faeces with spin columns. *Euk. J. Clin. Microbiol. Infect. Dis*. **19**(5): 358-361.

Verweij, J.J., Oostvogel1, F., Brienen1, E.A.T., Nang-Beifubah, A. Ziem, W. & Polderman, A.M.(2003). Short communication: Prevalence of *Entamoeba histolytica and Entamoeba dispar* in northern Ghana.*Trop. Med. Intern. Hlth.***8**: **1153**-1156.

Walsh, J.A. (1986). Problems in recognition and diagnosis of amoebiasis: Estimation of the global magnitude of morbidity and mortality. *Rev. Infec.Dis* **8:**228-238.

WHO/PAHO/UNESCO (1997). A consultation with expert on amoebiasis. Mexico City, *Epidemiol. Bullet*. **18**:97-100.

Yousef, M., Shurman, Bougnoux, M., Rawashdeh, M., Bretangne, S. & Strockbine, N.(2000).Bacterial viral and parasitic enteric pathogens associated with acute diarrhea in hospitalized children in northern Jordan. *Medi. Microbiol*. **28**:257-263.

Zaki, M., & Clark, C.G. (2001). Isolation and characterization of polymorphic DNA from *Entamoeba histolytica*. *J. Clin. Microbiol*. **39**: 897– 905.

Zaki, M., Meelu, P., Sun, W., & Clark.C.G. (2002). Simultaneous differentiations and typing of *Entamoeba histolytica* and *Entamoeba dispar*. *J.Clin. Microbiol*.**40**: 1271–1276.

9. Appendixes

Questionaire for *Entamoeba* species commonly Diagnosed as *Entamoeba histolytica* and the Enteric Bacteria in the Pathogenesis of Diarrhea in Addis Ababa.

LAB ID ☐

1. **PERSONAL IDENTIFICATION**

Card ID ☐ Sex ☐ Age ☐

Date ☐ ☐ ☐

2. **Address** ----------

3. **ENROLMENT CRITERIA**

DIARRHOEA: duration

< 1 week ☐ 1-4 weeks ☐ >4 weeks ☐

Frequency of diarrhea ----------

Supply of pure water 1. Yes ----- 2. No -----

Use of latrine 1. Yes ----- 2. No -----

4. **HISTORY**

Put ‘’right’’ make in the given boxes after identifying patients complaints/ signs and symptoms

CLINICAL HISTORY

	YES	NO
Watery	☐	☐
Mucoid/bloody diarrhea	☐	☐
Tenesmus	☐	☐
Constipation	☐	☐
Increased gas (flatulence)	☐	☐
Abdominal pain	☐	☐
Weight loss	☐	☐
Fever	☐	☐
Vomiting and Nausea	☐	☐
Other antibiotics taken	☐	☐

SIGNS AND SYMPTOMS

	YES	NO	NA
Liver tenderness	☐	☐	☐
Sweats	☐	☐	☐
Chills	☐	☐	☐
Vomiting	☐	☐	☐
Weakness	☐	☐	☐
Mild jaundice	☐	☐	☐
Sings of dehydration	☐	☐	☐

5. LAB INVESTIGATION REPORT

a. Macroscopy

i. Consistency (one choice)

Soft ☐

Watery ☐

Diarrhea ☐

ii. Appearance (one choice)

Bloody ☐

Mucoid ☐

Bloody/mucoid ☐

Watery ☐

Loose ☐

b. Microscopy

E. histolytical/ E.dispar

Cyst ☐ Trophozoite ☐

Others----------

6. Bacterial organisms isolated ----------

7. Susceptibility test 1= Amp;2=Cotri;3=Caf;4=Tet;5=Nor;6=Amp

Declaration

I, the undersigned declare, that this thesis is my original work and has not been presented for a degree in any other university. All the sources of materials in this thesis are correctly acknowledged.

Name Asnakew Worku

Signature _____

This Thesis has been approved by

Prof. Beyene Petros (Advisor) _____

Dr. Amha Kebede (Advisor) _____