In vitro sensitivity study of different brands of Ornidazole against clinical isolates of *Entamoeba histolytica*

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This Thesis paper is dedicated to my

Parents

The thesis paper on "*In vitro* sensitivity of different brands of Ornidazole against clinical isolates of *Entamoeba histolytica*" submitted to the Department Of Pharmacy, East West University, Mohakhali, Dhaka in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (B.Pharm) was carried out by Fahmia Afrin (ID: 2007-1-70-008)

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CERTIFICATE

This is to certify that the thesis "*In vitro* sensitivity of different brands of Ornidazole against clinical isolates of *Entamoeba histolytica* " submitted to the Department Of Pharmacy, East West University, Mohakhali, Dhaka in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (B.Pharm) is carried out by Fahmia Afrin (ID: 2007-1-70-008) under our guidance and supervision and that no Part of the thesis has been submitted for any other degree. We further certify that all the sources of information and laboratory facilities availed of this connection is duly acknowledged.

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ABSTRACT

Entamoeba histolytica is the etiological agent of amoebic dysentery and amoebic liver abscess. Amoebiasis is one of the most common health problems in the developing countries. Almost 10% people of the world are suffering from amoebiasis. Poverty, ignorance, overcrowding, poor sanitation and malnutrition favor transmission and increased disease burden. The main objective of the study was to find out the *in vitro* sensitivity of different brands of Ornidazole against clinical isolates of *Entamoeba histolytica*. *Entamoeba histolytica* isolates was collected from the stool sample and cultured in different culture medium. Different brands of Ornidazole were prepared in different concentrations of 4.6, 2.3 ,1.15 , 0.575 , 0.287 and 0.143 μM using the Di-methyl sulphoxide (DMSO) solvent and incubated at 24 hours respectively. After incubation the isolates of viable *Entamoeba histolytica* were calculated. Viable count of the *Entamoeba histolytica* in each concentration of Ornidazole (brands) were compared to the initial concentration and with the Ornidazole standard . After 24 hours of incubation concentration of 4.6 μM showed very few amounts of *Entamoeba histolytica*. As the concentrations of Ornidazole (brands and standard) decreased (4.6, 2.3 ,1.15 , 0.575 , 0.287 and 0.143 μM), after 48 hours of incubation the viability of clinical isolates of *Entamoeba histolytica increased*. These results showed that Ornidazole brands were effective to inhibit the growth of clinical isolates *Entamoeba histolytica*.

1.1 History

Humans harbor nine species of intestinal amoebae, of which only one, *Entamoeba histolytica*, is a pathogen. The life cycle is simple. The amoebae live and multiply in the gut and form cysts that are passed out in the feces and infect new individuals when they are consumed in contaminated water or food. Most infections are asymptomatic, but some strains of *E. histolytica* can invade the gut wall, causing severe ulceration and amoebic dysentery characterized by bloody stools. If the parasites gain access to damaged blood vessels, they may be carried to extraintestinal sites anywhere in the body, the most important of which is the liver, where the amoebae cause hepatic amoebiasis. Supposed evidence that both the intestinal and liver forms of the disease were recognized from the earliest times is circumstantial because there are so many causes of both the bloody dysentery characteristic of amoebiasis and the symptoms of hepatic amoebiasis that many of these records are open to other interpretations. With these reservations in mind, the earliest record is possibly that from the Sanskrit document *Brigu-samhita*, written about 1000 BC, which refers to bloody, mucose diarrhea . Assyrian and Babylonian texts from the Library of King Ashurbanipal refer to blood in the feces, suggesting the presence of amoebiasis in the Tigris-Euphrates basin before the sixth century BC , and it is possible that the hepatic and perianal abscesses described in both *Epidemics* and *Aphorisms* in the *Corpus Hippocratorum* refer to amoebiasis. Since epidemics of dysentery by itself are likely to result from bacterial infections and dysentery associated with disease of the liver is likely to be amoebic, later records are easier to interpret. In the second century AD, Galen and Celsus both described liver abscesses that were probably amoebic, and the works of Aretaeus, Archigenes, Aurelanus, and Avicenna toward the end of the first millennium give good accounts of both dysentery and hepatic involvement . As amoebiasis became widespread in the developed world, there were numerous records of "bloody flux" in Europe, Asia, Persia, and Greece in the Middle Ages . The disease appears to have been introduced into the New World by Europeans sometime in the 16th century , and with the later development of European colonies and increased world trade, there are numerous clear descriptions of both the intestinal and hepatic forms of amoebiasis. In the 19th century, several books mainly concerned with diseases in India, including Researches into the Causes, Nature and Treatment of the More Prevalent Diseases of India and of Warm Climates Generally by James Annersley, clearly refer to both intestinal and hepatic amoebiasis , and it is now generally agreed that this book contains the first accurate descriptions of both forms of

the disease. The connection between amoebic dysentery and liver abscesses was described by William Budd, the English physician who discovered the method of transmission of typhoid . The amoeba itself, *E. histolytica,* was discovered by Friedrich Lösch (also known as Fedor Lesh) in 1873 in Russia , and Lösch also established the relationship between the parasite and the disease in dogs experimentally infected with amoebae from humans. (Hollander D H; 1976)

1.2 Amoebiasis

Amoebiasis is one of the most common health problems in the world. It is estimated that annually about 480 million people develop clinical Amoebiasis and at least 40,000 die (Walsh, 1998). Invasive Amoebiasis generally induces a systemic humoral immune response demonstrable about 1 week after the onset of symptoms (Ortiz, 1990; Ortiz, 1980). Anti-amoebic antibodies persist even after invasive amoebiasis has healed or after subclinical amoebic infection has disappeared (Knobloch, 1983). Amoebiasis is an infectious disease caused by a one-celled parasite called *Entamoeba histolytica*, which causes both intestinal and extraintestinal infections. Two species of Entamoeba are morphologically indistinguishable: *Entamoeba histolytica* is pathogenic and *Entamoeba dispar* harmlessly colonizes the colon. Amoebas adhere to and kill the cells of the colon and cause dysentery with blood and mucus in the stool. Amoebas also secrete substances called proteases that degrade lining of the colon and permit invasion into the bowel wall and beyond. Amoebas can spread via the circulation to the liver and cause liver abscesses. The infection may spread further by direct extension from the liver or through the bloodstream to the lungs, brain, and other organs (Kumar, 1999). Amoebiasis can affect people living anywhere in the world, but is most common where living conditions are crowded or there is poor hygiene and sanitation. Amoebiasis is common in parts of Africa, Central America, South America, India and Southeast Asia. About 500 million people worldwide are believed to carry *Entamoeba histolytica* in their intestines. Amoebiasis is believed to cause between 40,000 and 100,000 deaths worldwide each year (Zaki, 2006 , Bansal, 2004).

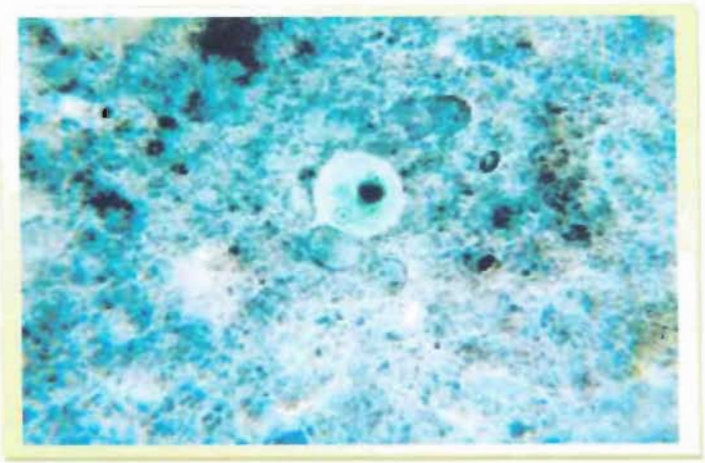

Figure 1: *E. histolytica* (Ivey M. H., 1961)

Table-1 Types Of Amoebiasis (Johnson et al,2000)

WHO Clinical Classification of Amoebiasis Infection (Modified)	**Pathophysiologic Mechanisms**
Asymptomatic infection	Colonization without tissue invasion
Symptomatic infection	Invasive infection
Intestinal amoebiasis	
A. Amoebic dysentery	Fulminant ulcerative intentional disease
B. Nondysentery gastroenteritis	Ulcerative intestinal disease
C. Amoeboma	Proliferative intestinal disease
D. Complicated intestinal amoebiasis	Perforation, hemorrhage, fistula
E. Post-amoebic colitis	Mechanism unknown
Extraintestinal amoebiasis	
A. Nonspecific hepatomegaly	Intestinal infection with no demonstrate invasion
B. Acute nonspecific infection	Amoebas in liver but without abscess

C. Amoebic abscess	Focal structural lesion
D. Amoebic abscess, complicated	Direct extension to pleura, lung, peritoneum or pericardium
E. Amoebiasis cutis	Direct extension to skin
F. Visceral amoebiasis	Metastatic infection of lung, spleen or brain.

1.3 The organism

4 species of Entamoeba:

Nonpathogenic: *E. dispar, E. coli, E. hartmanni*

Pathogenic: *E. histolytica*

Amoebiasis = A Parasitic infection caused by the protozoan (Entamoeba histolytica).

Factors contributing to faecal-oral

Poor education

Poverty and overcrowded

Unsanitary conditions

HIV infection (Cunnick C C , et.al , 1978)

1.4 Amoeba

An amoeba is a one-celled protozoan. Look for tiny gray "blobs" on the slide. An amoeba moves with pseudopods ("false feet"). It extends a "foot" of cytoplasm in one direction, and the rest of the body flows into that area. An amoeba surrounds another live organism with its pseudopods and takes it into a food vacuole. When an amoeba gets too large, it divides in half. The nucleus splits, too. Size is 600 μm (Two amoebas would almost fit in 1 mm.) Amoebae are unicellular organisms common in the environment: many are parasites of vertebrates and invertebrates. Relatively few species inhabit the human intestine and only *Entamoeba histolytica* is identified as a human intestinal pathogen. Amoeba itself is found in decaying vegetation in fresh and salt water, wet soil, and animals (Grath Mc.; 2001). Due to the ease with which they may be obtained and kept alive, they are common objects of study as representative protozoa and to demonstrate cell structure and function. The cell's organelles and cytoplasm are enclosed by a cell membrane, obtaining its food through phagocytosis. Amoebas have a single large tubular pseudopod at the anterior end, and several secondary ones branching to the

sides. The most famous species, *Amoeba proteus*, is 700-800 μm in length but the species *Amoeba dubia* is as large as a millimeter, and visible to the naked eye. Its most recognizable features include a single nucleus and a simple contractile vacuole to maintain osmotic pressure. Food enveloped by the amoeba is stored and digested in vacuoles. *Amoeba* reproduces through binary fission. In cases where the *amoeba* is forcibly divided, the portion that retains the nucleus will survive and form a new cell and cytoplasm, while the other portion dies (Cunnick C C; Diamond L S ; Harlow D R; 1978)

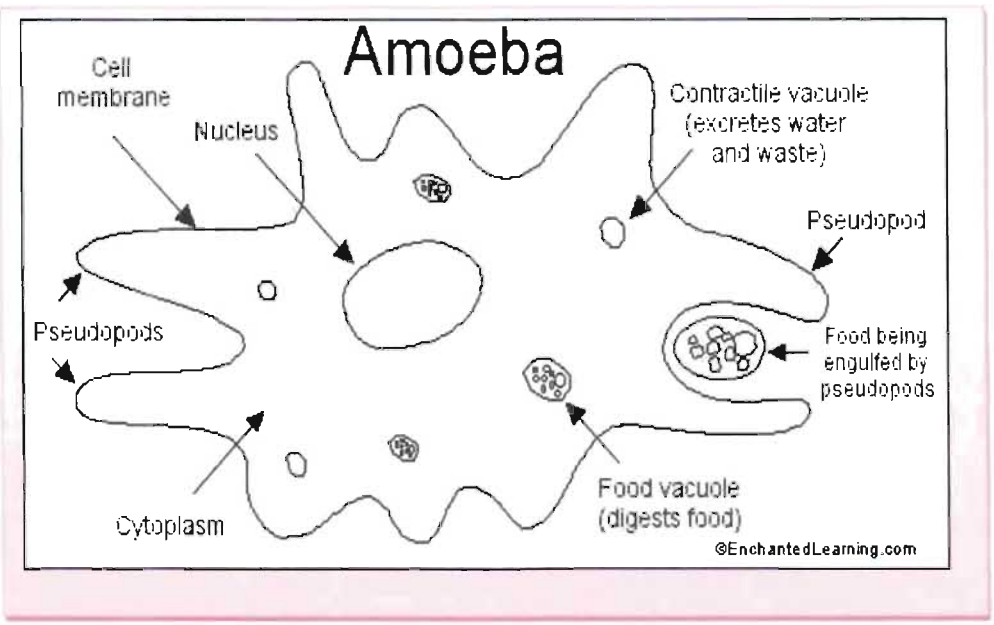

Figure 2: *Amoeba proteus* (www.wikipedia .com)

1.5 *Entamoeba histolytica*

Entamoeba histolytica is an anaerobic parasitic protozoan, part of the genus *Entamoeba*. Predominantly infecting humans and other primates. *E. histolytica* is estimated to infect about 50 million people worldwide. Previously, it was thought that 10% of the world population was infected, but these figures predate the recognition that at least 90% of these infections were due to a second species, *E. dispar*. Mammals such as dogs and cats can become infected transiently but are not thought to contribute significantly to transmission.

1.5.1 Scientific classification

Domain: Eukaryota

Phylum:	Amoebozoa
Class:	Archamoebae
Genus:	Entamoeba
Species:	*E. histolytica*

Entamoeba histolytica is an amoeboid protozoan parasite of the intestinal tract and in some cases other visceral organs especially the liver. There are several species in this genus, distinguished by their number of nuclei in the cyst and position of the endosome, whether or not they form a cyst, and whether they invade tissues or remain in the intestinal lumen.(Lyerly D, et al. , 2000)

1.5.2 Structure of *E histolytica*

E histolytica has a relatively simple life cycle that alternates between trophozoite and cyst stages The trophozoite is the actively metabolizing, mobile stage, and the cyst is dormant and environmentally resistant. Diagnostic concern centers on both stages . Trophozoites vary remarkably in size-from 10 to 60 μm or more in diameter, and when they are alive they may be actively motile. Amebas are anaerobic organisms and do not have mitochondria. The finely granular endoplasm contains the nucleus and food vacuoles, which in turn may contain bacteria or red blood cells. The parasite is sheathed by a clear outer ectoplasm. Nuclear morphology is best seen in permanent stained preparations. The nucleus has a distinctive central karyosome and a rim of finely beaded chromatin lining the nuclear membrane. The cyst is a spherical structure, 10-20 μm in diameter, with a thin transparent wall. Fully mature cysts contain four nuclei with the characteristic amebic morphology. Rod-like structures (chromatoidal bars) are present variably, but are more common in immature cysts. Inclusions in the form of glycogen masses also may be present. A number of non-pathogenic amoebae can parasitize the human gastrointestinal tract and may cause diagnostic confusion. These include *Entamoeba hartmanni, Entamoeba gingivalis, Entamoeba coli, Endolimax nana*, and *Iodamoeba butschlii*.(Lanuza M. D.et.al.1997)

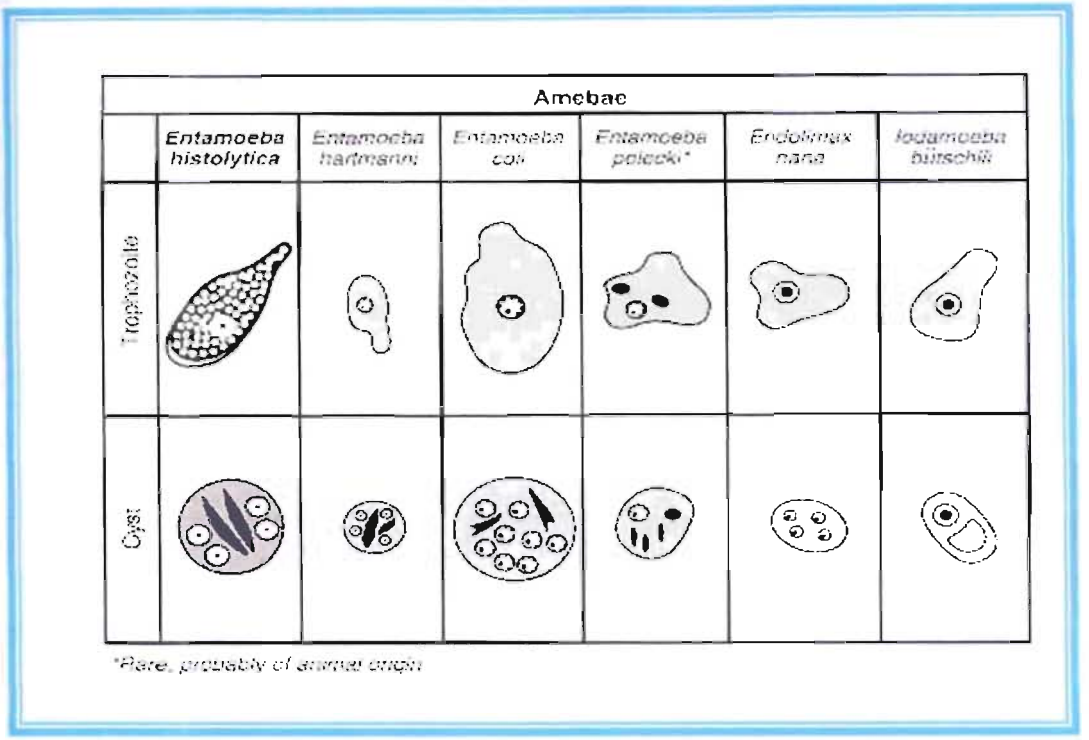

Figure 3 : Amoeba present in stool specimens of humans.(Melvin D.et.al.1969)

Table-2 Characteristics of *Entamoeba histolytica* (Ramamurti, 1973)

Genus and Species	*Entamoeba histolytica*
Etiologic Agent of	Amoebiasis; Amoebic dysentery; Extraintestinal Amoebiasis, usually Amoebic Liver Abscess = "anchovy sauce"); Amoeba Cutis; Amoebic Lung Abscess ("liver-colored sputum")
Infective stage	Cyst
Definitive Host	Human
Portal of Entry	Mouth
Mode of Transmission	Ingestion of mature cyst through contaminated food or water
Habitat	Colon and Cecum
Pathogenic Stage	Trophozoite
Locomotive apparatus	Pseudopodia ("False Foot")

Motility	Active, Progressive and Directional
Nucleus	'Ring and dot' appearance: peripheral chromatin and central karyosome
Mode of Reproduction	Binary Fission
Lab Diagnosis	Most common is Direct Fecal Smear (DFS) and staining (but does not allow identification to species level); Enzyme immunoassay (EIA); Indirect Hemagglutination (IHA); Antigen detection – monoclonal antibody; Polymerase Chain Reaction (PCR) for species identification. Culture: From faecal samples - Robinson's medium, Jones' medium
Trophozoite Stage	
Diagnostic Feature	Ingested RBC; distinctive nucleus
Cyst Stage	
Chromatoidal Body	'Cigar' shaped bodies (made up of crystalline ribosomes)
Number of Nuclei	1 in early stages, 4 when mature
Pathognomonic/Diagnostic Feature	'Ring and dot' nucleus and chromatoid bodies

1.6 Life cycle of *E. histolytica*

It has four nuclei in the cyst, a central endosome, forms a cyst, and can be a tissue invader. The amoeboid trophozoites can live in the intestinal crypts, feeding on intestinal contents and host tissue, and multiplying by fission. The trophozoites can be carried out in the faeces. As the faeces pass through the colon they dehydrate.The dehydration of the faeces causes the trophozoites to begin the process of encystment. Undigested food is discharged, and the trophozoite condenses and forms a spherical shape to form what is called the pre-cyst, and the cyst wall is secreted. Within the cyst there are two nuclear divisions resulting in 2 nuclei in the immature cyst and 4 nuclei within the mature cyst.

The cyst can resist desiccation for 1-2 weeks. When the cyst is ingested by another host the parasite excysts in the intestine and undergoes cytoplasmic division to produce 4 trophozoites. In some cases the trophozoites secrete Proteolytic enzymes which destroy the intestinal epithelium allowing the trophozoite to enter the host tissue. These can form large abscesses that may allow the parasite to enter the blood stream and be carried to the liver and other organs. In these extra-intestinal sites the trophozoites also can cause extensive tissue destruction (Meyer E. A. 1970).

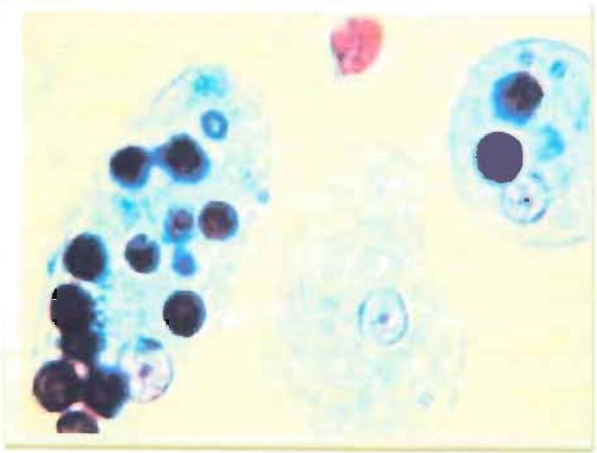

Figure 4 : The amoeboid trophozoites (Meyer E. A. 1970)

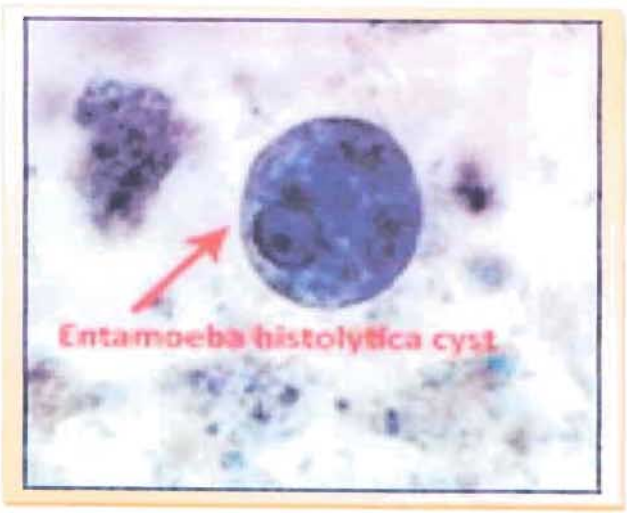

Figure 5: *Entamoeba histolytica* (Meyer E. A. 1970)

1.6.1 Transmission

The active (trophozoite) stage exists only in the host and in fresh loose feces; cysts Survive Outside the host in water, soils and on foods, especially under moist conditions

in the latter. The cysts are readily killed by heat and by freezing temperatures, and survive for only a few months outside of the host. When cysts are swallowed they cause infections by excysting (releasing the trophozoite stage) in the digestive tract. *E. histolytica*, as its name suggests (*histo-lytic* = tissue destroying), is pathogenic; infection can lead to amoebic dysentery or amoebic liver abscess. Symptoms can include fulminating dysentery, bloody diarrhea, weight loss, fatigue, abdominal pain, and amoeboma. The amoeba can actually 'bore' into the intestinal wall, causing lesions and intestinal symptoms, and it may reach the blood stream. From there, it can reach different vital organs of the human body, usually the liver, but sometimes the lungs, brain, spleen, etc. (Robinson G. L. 1968)

1.6.2 Genome

The genome data of *E. histolytica* have been reassembled and reannotated, incorporating significant structural and functional modifications to existing gene models. The 20 million basepair genome assembly contains 8,160 predicted genes; known and novel transposable elements. (Robinson, G. L. 1968)

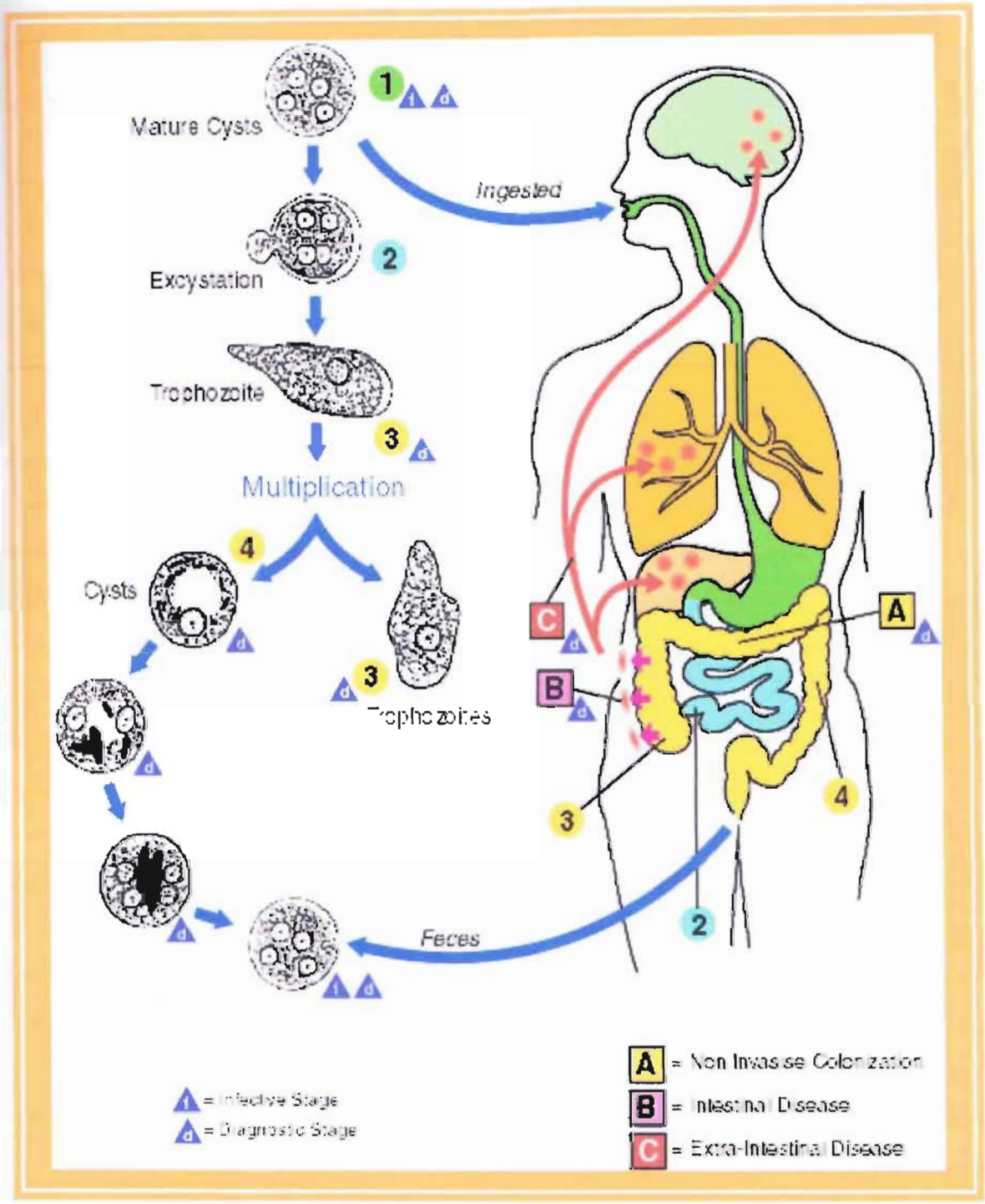

Figure 6: Life-cycle of *Entamoeba histolytic* (Philliasian B. P. 2001)

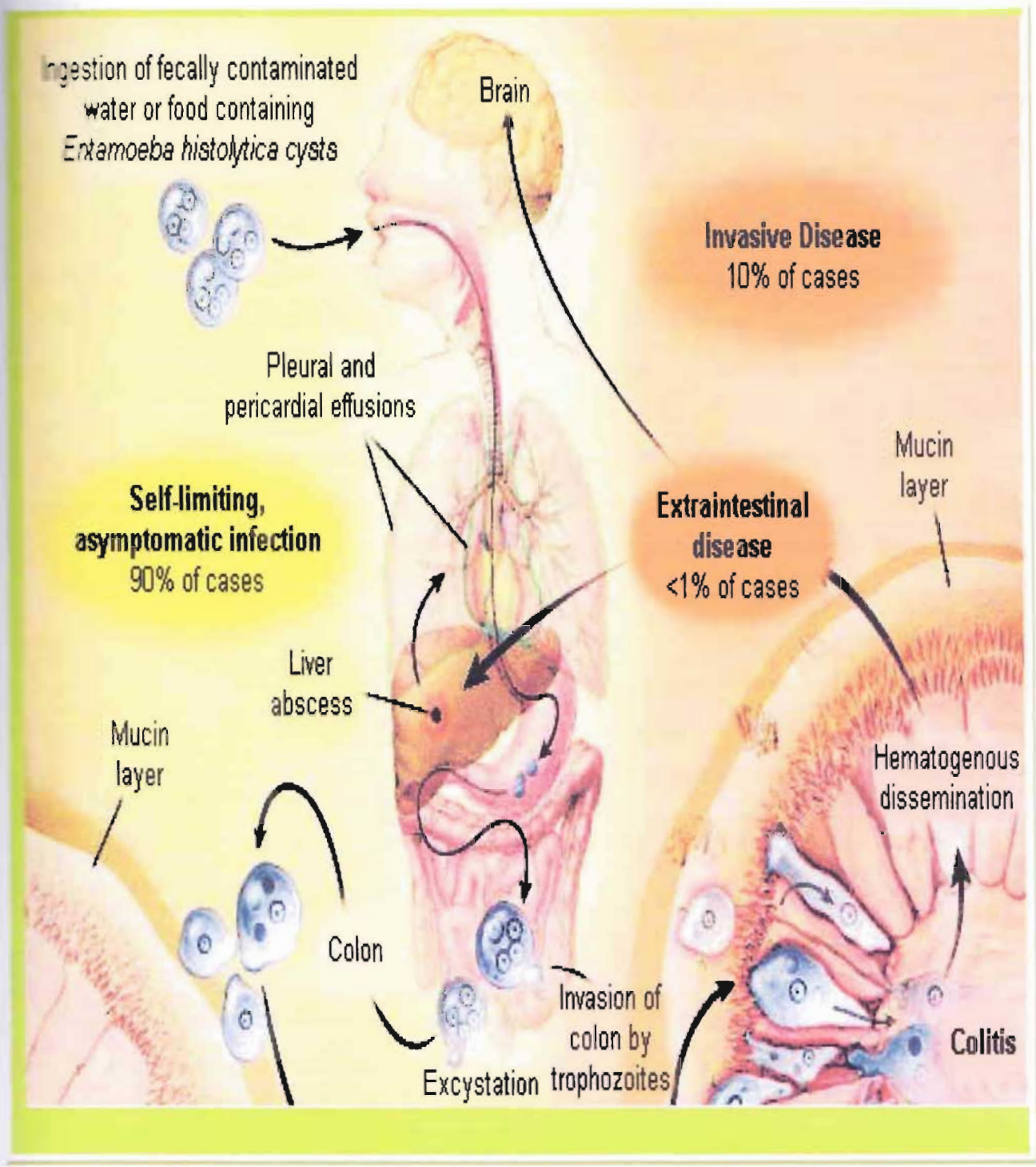

Figure 7 : Multiplication and life cycle of *E histolytica* & it's effect(Philliasian B. P. 2001)

1.7 Pathogen interaction

E. histolytica may modulate the virulence of certain human viruses and is itself a host for its own viruses. For example, cells are infected with HIV often consumed by *E.*

histolytica. Infective HIV remains viable within the amoeba, although fortunately there has been no proof of human re-infection from amoeba carrying this virus. A burst of research on viruses of *E. histolytica* stem from a series of papers published by Diamond et al. from 1972 to 1979. In 1972, they hypothesized two separate polyhedral and filamentous viral strains within E. histolytica that caused cell lysis. Perhaps the most novel observation was that two kinds of viral strains existed, and that within one type of amoeba (dubbed HB-301) the polyhedral strain had no detrimental effect but led to cell lysis in other (dubbed HK-9) strains (Samuels R. ,2000).

1.9 Pathogenesis

The aspects of pathogenesis which have been investigated experimentally can be broadly categorized into mechanisms involving

(i) interactions with the intestinal flora,
(ii) lysis of target cell by direct adherence,
(iii)lysis of target cell by release of toxins and
(iv)phagocytosis of target cells

Colonization and interaction with the intestinal flora

In the gut the trophozoites are constantly interacting with the intestinal flora. Studies have shown that trophozoites undergo changes on interacting with bacteria. (Yap E H, 2000).

Adherence to establish direct contact between trophozoite and target cell

Adherence of trophozoites to target cells is a necessary prerequisite for cytotoxicity. Two surface molecules responsible for adherence have been identified—one inhibitable by galactose or N-acetyl-D-galactosamine (GalNAc) and the other inhibitable by N-acetyl-D-glucosamine (GlcNAc) polymers. The following data suggest that this molecule plays an essential role in amoebic adherence to target cells - binding of trophozoites to cells was inhibited 90-95% by 50 mM galactose and GalNAc while other sugars had no effect (Yap E H, 2000).

Lysis of target cells by release of toxins and introduction of membrane channels

Prior to mucosal invasion by *E. histolytica* there is depletion of mucous and disruption of epithelial barrier. As the tropozoites adhere with the target cell then Ca2+ influx increases. This causes the release of cytotoxic enzymes and toxins for example- phospholipase. The protease enzyme helps to degrade the cell layer so that tropozoits can excess into the cell (Yap E H , 2000).

Phagocytosis

Trophozoites from stools of many infected patients contain ingested erythrocytes and have much higher rate of erythrophagocytosis than healthy human carrier.(Moe K T ; 1996)

1.10 Defending mechanism of body against *E histolytica*

The sensing of Pathogen Associated Molecular Patterns (PAMPS) by innate immune receptors, such as Toll-like receptors (TLRs) is the first step in the inflammatory response to pathogens. E. histolytica ,the etiological agent of amoebiasis has a surface molecule with the characteristic of a PAMP. This molecule is termed lipopeptidophosphoglycan (LPPG), is recognized through TLR2 and TLR4 and release cytokines from human monocytes, macrophages and dendritic cell. LPPG activates dendretic cell which increases the expression of costimulatory molecules.LPPG activates NKT cell. And it limits amoebic liver abscess development. LPPG also increases antibody production. Because it is recognized by both the innate and adaptive innune system. (Samuel R. 2000).

Figure 8 : Host defense against *E. histolytica*.(Mann B.J.; 2003)

1.11 Signs and Symptoms

The clinical effects of amoebiasis vary with the severity of the infaction. Acute amebic dysentery causes a sudden high temperature of 104° to 105° F (40° to 40.6° C) accompanied by chills and abdominal cramping; profuse, bloody, mucoid diarrhea with tenesmus; and diffuse abdominal tenderness due to extensive rectosigmoid ulcers.Chronic amoebic dysentery produces intermittent diarrhea that lasts for 1 to 4 weeks and recurs several times a year. Such diarrhea produces 4 to 8 (or, in severe diarrhea, up to 18) foul-smelling mucus- and blood-tinged stools daily in a patient with

a mild fever, vague abdominal cramps, possible weight loss, tenderness over the caecum and ascending colon and, occasionally, hepatomegaly. Amebic granuloma produces blood and mucus in the stool and, when granulomatous tissue covers the entire circumference of the bowel, causes partial or complete obstruction. Parasitic and bacterial invasion of the appendix may produce typical signs of subacute appendicitis (abdominal pain and tenderness). Occasionally, *E. histolytica* perforates the intestinal wall and spreads to the liver. When it perforates the liver and diaphragm, it spreads to the lungs, pleural cavity, peritoneum and, rarely, the brain. (Kobiler D , 1999)

1.12 Diseases

Amoebiasis is first and foremost an intestinal disease. Contamination occurs via the digestive tract, the colon being the seat of infection.

Amoebic liver disease: This is the most common form of invasive Amoebiasis. This can be found in all age groups, but are 10 times more frequent in adults than in children. Fever is present in most cases; it varies between 38-40^{O}C, Anorexia, Weight loss, Vomiting and Fatigue may all be present as symptom (Gordon, 2009).

Peritoneal amoebiasis: This is caused by the rupture of a hepatic liver abscess. It is characterized by sudden increase in abdominal pain (Gordon, 2009).

Pericardial amoebiasis: Pericardial involvement is the most serious complication of an amoebic liver abscess. It occurs in less than 1% of all amoebic liver abscesses, especially of the left lobe (Gordon, 2009).

Pleuropulmonary amoebiasis: Invasion of the pleural cavity is most commonly due to extension from a liver abscess and occurs in <1% of those with amoebic dysentery. The first clinical symptoms are those of the liver abscess, followed by severe pain in the lower chest, often radiating to the right shoulder, there may be non productive cough (Gordon, 2009).

Cerebral amoebiasis: Symptoms depend on the site and size of the lesion as many as 50% of patients may have abrupt onset of symptoms and die from cerebellar involvement

or rupture within 12-72hours (Gordon, 2009).

1.13 Clinical manifestation

Acute amoebic dysentery

- Slight attack of diarrhea, altered with periods of constipation and often accompanied by tenesmus.
- Diarrhea, watery and foul smelling stool often containing blood-streaked mucus
- Nausea, flatulence, abdomnal distension and tenderness in the right iliac region over the colon (Kobiler D.; 1999).

Chronic amoebic dysentery

- Attack dysentery that lasts for several days, usually succeeded by constipation
- Tenesmus accompanied by the desire to defacate
- Anorexia, weight loss, and weakness
- Liver may be enlarged
- The stool at first is semifluid but soon becomes watery, bloody, and mucoid
- Vague abdominal distress, flatulence, constipation or irregularity of bowel
- Abdomen loses its elasticity
- On sigmoidoscopy, scattered ulceration with yellowish (Kobiler D ; 1999).

Extraintestinal forms

Hepatic

- Pain at the upper right quadrant with tenderness of the liver
- Abscess may break through the lungs, patient coughs anchovy-sauce sputum
- Jaundice

- Intermittent fever
- Loss of weight or anorexia (Kobiler D; 1999)

1.14 Epidemiology

About 10% of the world's population is infected with *E. histolytica*, and 10% of those infected go on to develop symptoms of the disease. Although the numbers may appear small, amoebiasis is actually the second leading parasitic disease worldwide. Although prevalence of the disease is worldwide, amoebiasis is most common in people in developing countries where there is poor sanitation and higher rates of contamination of food and water. The poor in developing countries encounter amoebiasis on a daily basis . In the United States, amoebiasis is most often found in immigrants from developing countries. For instance, in New York, the comparatively higher rates of immigration lead to about 500 cases reported each year . As well, people who have traveled to developing countries or who live in places of poor sanitation have a higher chance of getting infected. Men engaged in homosexual intercourse can also become infected, but they generally do not develop symptoms. Although there are variations in prevalence of the disease from country to country, amoebiasis occurrence is generally associated with places of poor sanitation, where the *E. histolytica* cysts can be transmitted from person to person —mainly, high incidences are in tropical countries like India, Mexico, Central and South America . The prevalence of amoebiasis in underdeveloped countries reflects the lack of adequate sanitary systems (Smedley S R.; 1998)

1.15 Pathophysiology

1.15.1 Laboratory Diagnosis

Stool exam (cyst, white and yellow pus with plenty of amoeba)

Blood exam (Leukocytosis)

Proctoscopy/Sigmoidoscoppy

Diagnosis of amoebiasis can be very difficult. One problem is that other parasites and cells can look very similar to *E. histolytica* when seen under a microscope. Therefore, sometimes people are told that they are infected with *E. histolytica* even though they are

not *Entamoeba histolytica* and another amoeba, *Entamoeba dispar*, which is about 10 times more common, look the same when seen under a microscope. Unlike infection with *E. histolytica*, which sometimes makes people sick, infection with *E. dispar* does not make people sick and therefore does not need to be treat. Most laboratories do not yet have the tests that can tell whether a person is infected with *E. histolytica* or with *E. dispar*. Until these tests become more widely available, it usually is best to assume that the parasite is *E. histolytica*. A blood test is also available but is only recommended when the health care provider thinks that infection may have spread beyond the intestine (gut) to some other organ of the body, such as the liver. However, this blood test may not be helpful in diagnosing of current illness because the test may still be positive if one had amoebiasis in the past, even if someone is no longer infected now (Keene W E ;1986)

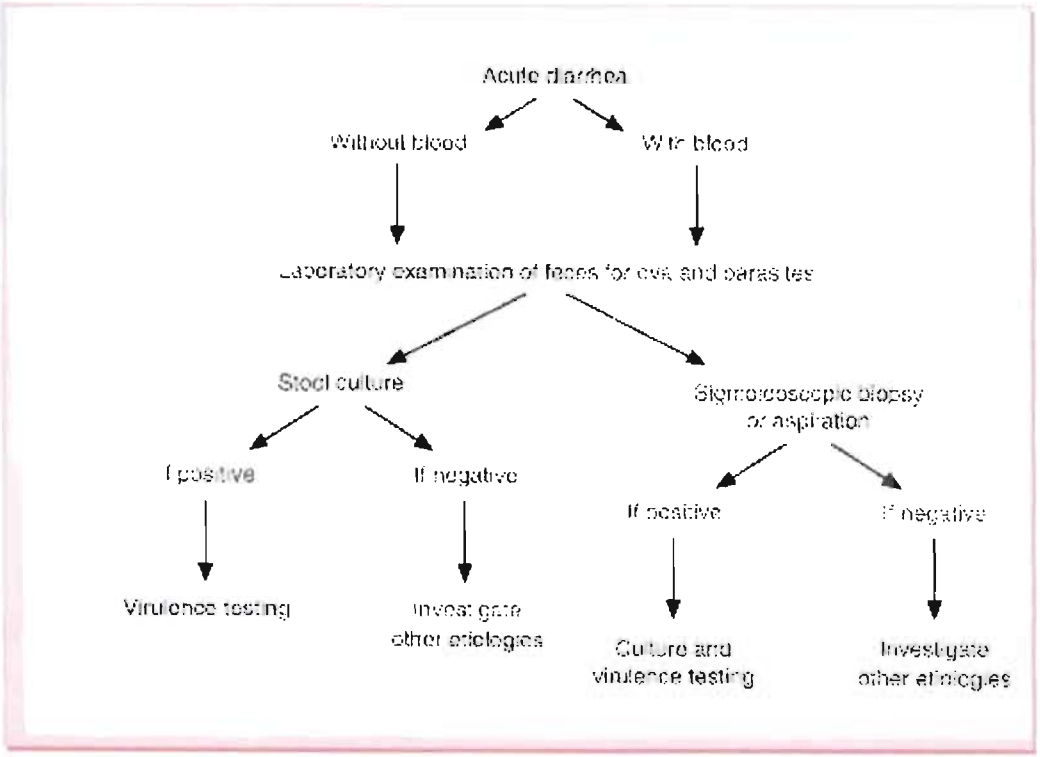

Figure 9 : Evaluation of suspected cases of intestinal amoebiasis(Keene W E ; 1986.)

1.15.2 Laboratory Diagnosis of Intestinal Amoebiasis

In cases of amoebiasis, stool samples are always heme-positive.Examination of a fresh stool smear for trophozoites containing ingested red blood cells is a rather insensitive

method. Routine microscopy cannot distinguish the *E. dispar* and *E. moshkovskii* non-pathogenic amoebae from *E. histolytica.* An enzyme immunoassay kit designed specifically to detect *E. histolytica* in fresh stool specimens is commercially available. PCR-based diagnostic tests have been developed, but are not widely available. Field studies that directly compared PCR with stool culture or antigen-detection tests for the diagnosis of *E. histolytica* infection suggest that these three methods are comparable. Colonoscopy and biopsy, or scraping at the margin of a colonic mucosal ulcer, provide valuable materials for diagnostic information.The mucosal lining between ulcers appears normal at colonoscopy. Histopathological findings include non-specific mucosal thickening and focal ulcerations with or without amoeba in a diffusely inflamed mucosal layer. (Maraha 2000).

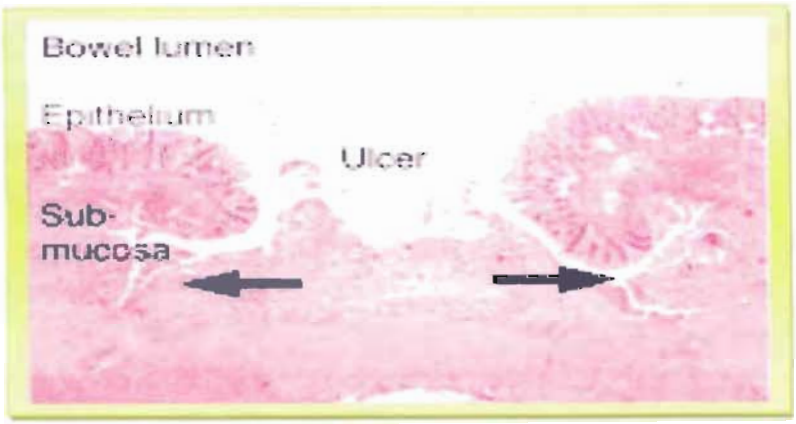

Figure 10 : Flask-shaped" ulcer of invasive intestinal amebiasis (Standly L ; 2009)

1.15.3 Laboratory Diagnosis of Amebic Liver Abscess

Laboratory findings include leukocytosis without eosinophilia in 80% of cases, mild anemia in more than half, elevated alkaline phosphatase levels in 80%, elevated transaminase levels in more aggressive disease, mild elevation of serum bilirubin level, and a high erythrocyte sedimentation rate.21,23,31 Abdominal ultrasonography, computed tomography (CT), and magnetic resonance imaging (MRI) are all excellent for detecting liver abscesses, but cannot distinguish amebic from pyogenic abscesses. Anti-amebic antibodies are present in up to 99% of patients who have been symptomatic for over a week.21,23 Serological examination should be repeated a week later in those with negative test on presentation. The galactose lectin antigen is present in the serum of

75% of subjects with amebic liver abscess, and may be particularly useful in patients presenting acutely, before an IgG serum anti-amebic antibody response occurs.32,33 Aspiration of the abscess is occasionally required to rule out a pyogenic abscess. Aspiration of amebic liver abscess yields an anchovypaste- like material that lacks white blood cells (WBCs) due to lysis by the parasite. Amebae are visible in the abscess fluid in a minority of patientswith amebic liver abscess. Fewer than half of patients with amebic liver abscess have parasites detected in their stool by antigen detection (Maraha , 2000).

1.16 Key diagnostic factors

Presence of risk factors (common) (Wiese , 2000). Patient may have had diarrhoea for 1 week or more at the time of presentation. *Entamoeba histolytica* diarrhea is usually lacking blood or mucus and is therefore indistinguishable from diarrhoea caused by a variety of other enteropathogens .(Wiese , 2000).Less than 50% of patients with a liver abscess will have diarrhoea at time of presentation, although a past history of diarrhoea or dysentery is common (Wiese , 2000).

1.17 Other diagnostic factors

Generalised abdominal pain (common)

- Present in most patients with amoebiasis.

Weight loss (common)

- About half of patients may report weight loss due to the subacute nature of the disease.

Cough (common)

- Common in patients with liver abscess

Fever (uncommon)

- Rare in intestinal infections but common in extraintestinal infections, such as liver and brain abscesses.

Altered mental status (uncommon)

- Present with amoebic brain abscess.

Dyspnoea (uncommon)

- Extension of liver abscess causing pleural or pericardial effusion.

Jaundice (uncommon)

- More common with pyogenic than amoebic liver abscess. (Wiese , 2000)

1.18 Nursing Management

Observe isolation and enteric precaution provides health education and instruct patient to

- Boil water for drinking or use purified water
- Avoid washing food from open drum or pail
- Cover leftover food
- Wash hands after defacation and before eating
- Avoid ground vegetables (lettuce, carrots, and the like) (Luaces A L ; 1988)

1.19 Spread of amoebiasis

Amoebiasis occurs when *Entamoeba histolytica* parasites are taken in by mouth and the most common way this happens is by person-to-person spread. Amoebiasis can also be spread by:

- Drinking contaminated water .

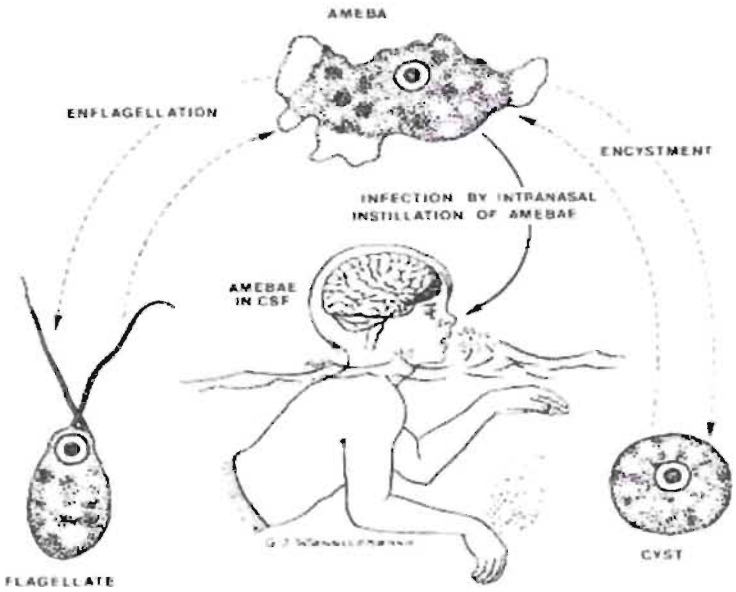

Figure 11 : Direct contact and transmission of the microorganisms through contaminated water (Luaces A L ;1988)

- Eating contaminated raw vegetables and fruit.
- Unprotected oral-anal sexual contact.
- Eating contaminated raw vegetables and fruit .
- Contaminated hands can spread the parasites to food that may be eaten by other people and surfaces that may be touched by other people. (Luaces A. L.; 1988)

1.20 Natural Home Remedies For the Treatment of Amoebiasis

Need to remove the kernal from the dried seeds of the bael fruit, and also the mango seed kernal. An equal quantities of these two kernels are taken. A teaspoonful of each need to ground together. One fourth of the mixture should be taken with a cup of rice gruel once a day for 3- 4 days. Helps to cure the amoebiasis. A once a week ripe bael sherbet is taken keeps the bowels in a healthy condition.Roast unripe bael fruit on a open is fired and removed its pulp. This should be eaten with a little jaggery or a sugar, a teaspoonful daily is especially beneficial for blood diarrhoea with mucus. The jam is made with the semi ripe or unripe bael fruit - a teaspoonful on a empty stomach every morning keeps the digestive system healthy. It helps in curing the amoebiasis. (Luaces A L; 1988)

1.21 Methods of Prevention

Health education

Sanitary disposal of feces

Protect, chlorinate, and purify drinking water

Detection and treatment of carriers

Fly control (they can serve as vector) (Said-Fernandez,1988).

1.22 Control

Preventive measures are limited to environmental and personal hygiene. Treatment depends on drug therapy, which in the case of some abscesses must be supplemented with drainage, either open or by aspiration. Effective drugs are available for liver abscess but intestinal infection is less successfully treated. No single drug is completely effective in eradicating amoebias from the gut, so reliance is often placed on combination therapy. It is also essential to pay concern on taking food suppliments. (Said-Fernandez, 1988)

1.23 Treatment

WHO/PAHO recommendations state that, when possible, *E histolytica* should be differentiated from morphologically similar species and treated appropriately. Given the small but substantial risk of invasive disease and the potential to transmit the infection to others, WHO/PAHO recommends treating all cases of proven *E histolytica*, regardless of symptoms. If *E. dispar* is the only species identified, then no treatment should be given and other causes should be sought as appropriate. The medications recommended to treat confirmed amoebiasis vary with clinical manifestation. Asymptomatic intestinal infection with *E. histolytica* should be treated with luminal amoebicides, such as paromomycin and diloxanide furoate. These medications will eradicate the luminal amebae and prevent subsequent tissue invasion and spread of the infection through cysts. Paromomycin, more widely available in the United States, has the advantage of not being absorbed in the bowel. Abdominal cramps and nausea are the most commonly reported adverse effects. A 10-day course at 30 mg/kg per day (divided into 3 daily doses) is typical. Some recommend follow-up stool examination to confirm eradication of cysts .Like amebic colitis, ALA typically responds well to a 5- to 10-day course of

metronidazole, which should also be followed with a luminal amoebicide. Amoebae rarely disseminate beyond the portal circulation. Given the small number of cases, no definitive treatment guidelines are available for management of extra intestinal, extrahepatic disease. As mentioned previously, infections with *E dispar* do not require treatment. Less is known about *E moshkovskii,* but it is likely that this infection also would not require treatment in most cases (Pritt, 2008).

Table 3: Types of drugs for medication (Gonzales MLM et.al, 2009)

Luminal	Arsenical compounds	Carbarsone, acetarsone or acetarsol, treparsol, diphetarsone, glycobiarsol or bismuth glycolylarsanilate, stovarsol, and thioarsenite, thiocarbarsone or thiocarabazone, arsthinol
	Hydroxyquinoline derivatives	Chiniofon or quinoxyl, clioquinol or iodochlorhydroxyquin, and iodoquinol or diiodohydroxyquin
	Dichloroacetamide derivatives	Diloxanide furoate or entamide furoate, clefamide, eticlordifene or ethylchlordiphene or etofamide or etophamide, and quinfamide
	Benzylamine derivatives	Teclozan, chlorbetamide or mantomide, and chlorphenoxamide or mebinol
	Antibiotic amoebicides	Tetracycline, oxytetracycline, chlortetracycline, erythromycin, paromomycin, and Fumagillin
	Nithrothiazole salicylamide	Nitazoxanide
Tissue	Emetine and its derivatives	Emetine hydrochloride, emetine bismuth iodide, dehydroemetine dihydrochloride, and dehydroemetine resinate
	Aminoquinoline	Chloroquine

	Thiazole derivative	Niridazole
	Nitroimidazoles	Metronidazole, tinidazole, ornidazole.

Table-4 Suggested Food Supplements

SL	Supplement	Daily Dosage	Comments
1	Vitamin-C	03/day	Till complete relief
2	Vitamin-E with Selenium	01/day	Till complete relief
3	Multivitamin	01/day	Life time

1.23.1 Metronidazole

Metronidazole is a prodrug. It is converted in anaerobic organisms by the redox enzyme pyruvate-ferredoxin oxidoreductase. The nitro group of metronidazole is chemically reduced by ferredoxin (or a ferredoxin-linked metabolic process) and the products are responsible for disrupting the DNA helical structure, thus inhibiting nucleic acid synthesis. Metronidazole is selectively taken up by anaerobic bacteria and sensitive protozoal organisms because of the ability of these organisms to reduce metronidazole to its active form intracellularly (Wikipedia-*Entamoeba histolytica*) (Burton, 2005).

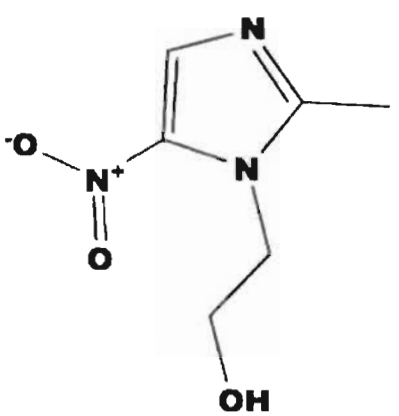

Fig 13: Chemical structure of metronidazole (www.wikipedia.com)

1.23.2 Emetine

The identification of emetine as a more potent agent improved the treatment of amoebiasis. While use of emetine still caused nausea, it was more effective than the crude extract of ipecac root. Additionally, emetine could be administered hypodermically which still produced nausea, but not to the degree experienced in oral administration. Although it is a potent anti-protozoal, the drug also can interfere with muscle contractions, leading to cardiac failure in some cases. Because of this, in some uses it is required to be administered in a hospital environment so that adverse events can be addressed. It is highly toxic. It can produce local irritant, pain, stiffness, eczematous lesions at the site of injection also it can produce nausea, vomiting, cramps, diarrhea, weakness and stiffness of muscles, hypotension, tachycardia, ECG changes, myocarditis (Burton, 2005).

1.23.3 Chloroquine

- Kills trophozoites, highly concentrated in liver.
- Used in hepatic amoebiasis.
- Completely absorbed from upper intestine and not so highly concentrated in intestinal wall – neither effective in invasive dysentery nor in controlling the luminal cycle.
- Efficacy similar to emetine but duration of treatment is longer and relapses are more frequent.
- Only used if metronidazole is not effective.
- A luminal amoebicide must always be given with or after chloroquine to abolish the luminal cycle.
- Dose : 600mg for 2d followed by 300mg daily for 2-3 wks (Burton, 2005).

1.23.4 Diloxanide Furoate

- Highly effective luminal amoebicide.
- Directly kills trophozoites.
- Furoate ester is hydrolyzed in intestine then released diloxanide.
- Diloxanide is a weaker amoebicide than furoate ester.

- Primarily metabolized by glucoronidation, excreted in urine.
- No antibacterial effect.
- Dose : 500mg (Burton, 2005).

1.23.5 Diiodohydroxyquin

It is effective in amoebicide and commonly used with metronidazole. 90% of the drug is retained in intestines and excreted in feces. It half life is 11-14 h. It is excreted in urine as glucuronides. (Burton, 2005).

1.23.6Tinidazole

Tinidazole may be a therapeutic alternative in the setting of metronidazole tolerance. Tinidazole may also be used to treat or prevent a variety of other bacterial infections, including *Helicobacter pylori*. The most common side effects reported with tinidazole are upset stomach, bitter taste, and itchiness. Other side effects which occur are headache and dizziness (Burton, 2005).

1.23.7Ornidazole

Ornidazole is a nitroimidazole antiprotozoal agent used in ameba and trichomonas infections. It is partially plasma-bound and also has radiation-sensitizing action. Somnolence, headache, nausea, vomiting, dizziness, tremor, rigidity, poor coordination, seizures, tiredness, vertigo, temporary loss of consciousness and signs of sensory or mixed peripheral neuropathy, taste disturbances, skin reactions (Burton, 2005).

1.23.8 Secnidazole

Congener with same spectrum of activity and potency.

- Oral absorption is rapid and complete.
- Metabolism is slower and it's half life is t ½ - 17-29h.

After 48h, single dose of 2g of Secnidazole remains w/n range of MIC values. Single dose of 2g has been reported to yield cure rates equal to multiple doses of previous 2 drugs (Burton, 2005).

1.24 Prevention

Food and water contaminated with feces containing the cysts of *E. histolytica* are the most common vehicles for transmission. Prevention, therefore, depends on interruption of the contamination-in gestation cycle. One such measure is the boiling or iodination of drinking water in endemic areas. In many areas, fruits and vegetables become contaminated when human excrement is used as fertilizer. Transmission of *E. histolytica* by infected food handlers can be controlled by local ordinances requiring periodic physical examinations, including stool examination, for all food handlers (Burton, 2005).

1.25 Incidence of Amoebiasis (Extrapolated Statistics)

Table-5 Southern Asia (US Census Bureau, Population Estimates, 2008)

Area	Incidence in percent	Population in area or density
Afghanistan	189	28,513,677[2]
Bangladesh	937	141,340,476[2]
Bhutan	14	2,185,569[2]
India	7,062	1,065,070,607[2]
Pakistan	1,055	159,196,336[2]
Sri Lanka	131	19,905,165[2]

Table-6 Southeastern Asia (US Census Bureau, Population Estimates, 2008)

Country	Incidence in percent	Population in area or density
East Timor	6	1,019,252[2]
Indonesia	1,581	238,452,952[2]
Laos	40	6,068,117[2]
Malaysia	155	23,522,482[2]
Philippines	571	86,241,697[2]
Singapore	28	4,353,893[2]
Thailand	430	64,865,523[2]
Vietnam	548	82,662,800[2]

1.26 Anti amoebic drug

Antiamoebic agents are drug used to treat amoebiasis. The term 'amoebiasis' usually refers to an infection caused by *E histolytica*. This parasitic diseases is one of the major cause of illness and death in many countries. In tropical country where more than 10% of the population may be infected in endemic areas. However it can also occur in temperature climates wherever standards of personal anf environmental hygiene are low. Even in the US 2 to 5% of the population is infected by *E. histolytica* in areas with poor sanitation (Smith R. F. 2007).

1.27.1 Ornidazole

Nitroimidazole drugs have been used for over 20 years, not only as major antimicrobial drugs but also as sensitizers of hypoxic tumors in conjunction with radiotherapy, thus possessing a wider spectrum of useful clinical activity than any other antibiotics . Ornidazole 5-nitroimidazole derivative. It is an anti-microbial agent. It is used in the treatment of susceptible protozoal infections and in the treatment and prophylaxis of anaerobic bacterial infections. The mean ornidazole elimination half-life is 12 hours, significantly longer than that of some nitroimidazole derivatives. This is a particular advantage for reducing the dosage frequency and duration of therapy in many of the relevant clinical .infections. It has been used for amoebic liver abscess, duodenal ulcers, giardiasis, intestinal lambliasis. (Smith R. F. 2007).

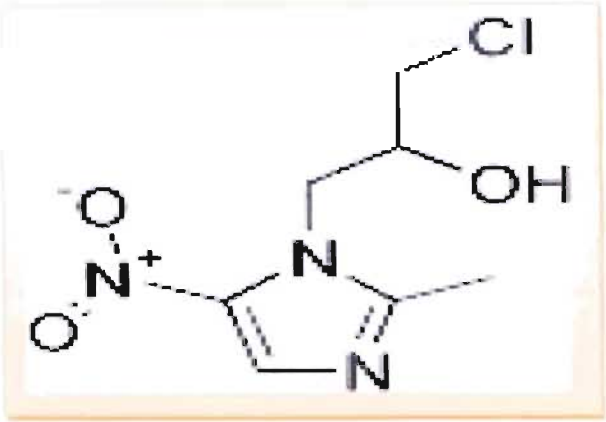

Figure 14: Structure of ornidazole (www.wikipedia .com)

Name : Ornidazole

Synonyms : 1(3-chloro-2-hydroxypropyl)-2-methyl-5-nitro imidazole; alpha (chloromethyl)- Methyl- 5- nitro-1H-Imidazole-1-ethanol

Physicochemical Properties : Ornidazole has a heterocyclic structure consisting of a nitroimidazole-based nucleus with a 2-hydroxy-3- chloro-propyl group in position 1 and a methyl group in position 2. It is synthesized from 5-nitroimidazole derivatives.

Molecular Formula : $C_7H_{10}ClN_3O_3$

Molecular Weight : 219.63 .(Smith R. F. 2007)

Appearance : It is a white to yellowish microcrystalline powder,

Solubility: Ornidazole is soluble in water, ether, ethanol and chloroform (Smith R. F. 2007).

Melting point: with a melting point between 358-360 K.

pH: It is 1% aqueous solution has a pH of approximately 6.6.

Half life: The mean half-life of elimination from human plasma is 11 to 14 hours. The long serum half-life of Ornidazole would permit a more convenient dosage interval, and it has an even greater antimicrobial activity compared with other nitroimidazole derivatives. Ornidazole has a large spectrum of activity and can be given in different dosage forms (Krishnainaya K. H. 1982).

Drug Interactions : Potentiates effect of oral anticoagulants. Prolongs the muscle-relaxant effect of vecuronium bromide (Smith R. F. 2007).

Adverse Drug Reactions : Headache, nausea, vomiting, dizziness, tremor, rigidity, poor coordination, seizures, tiredness, vertigo, temporary loss of consciousness and signs of sensory or mixed peripheral neuropathy taste disturbances , skin reactions.

Special Precautions : Renal and hepatic impairment, CNS diseases e.g. epilepsy or multiple sclerosis , pregnancy and lactation (Smith R. F. 2007).

Distribution : It is widely distributed in body tissues and fluids, including the cerebrospin fluid.

Route of administration: Ornidazole is administered orally, vaginally, or intravenously. Ornidazole is given by mouth in tablets after food, or intravenously. When given intravenously, solutions of ornidazole should be diluted to 5 mg or less per ml and 100 or 200 mL infused over 15 to 30 minutes. (Krishnainaya K. H. 1982).

Tolerability: Local and systemic tolerability of Ornidazole was excellent in humans when used in pregnancy, and patients showed complete remission without premature delivery.

Side effect: Ornidazole is well tolerated, with the most common side effects being nausea, abdominal pain, vertigo, headache, diarrhea, flatulence, and skin rash (Krishnainaya K. H. 1982).

1.27.2 Mechanism of action

5-Nitroimidazoles belong to the nitroheterocyclic family of compounds widely used for the treatment or prophylaxis of infections due to anaerobic bacteria and protozoa. They have also received much attention in cancer therapy as radiosensitizers of hypoxic tumors and by their direct cytotoxic effects towards hypoxic cells. Nitroimidazoles are thought to produce their bactericidal activity through four phases:

(I) entry into the bacterial cell
(II) nitro group reduction
(III) action of the cytotoxic by products
(IV) production of inactive end products

Bactericidal activity appears to be dependent on the formation of a redox intermediate metabolite in the bacterium. This toxic metabolite may interact primarily with DNA, RNA or intracellular proteins; however, its main effects are DNA strand breakage, inhibited repair and ultimately disrupted transcription and cell death (Smith R. F. 2007).

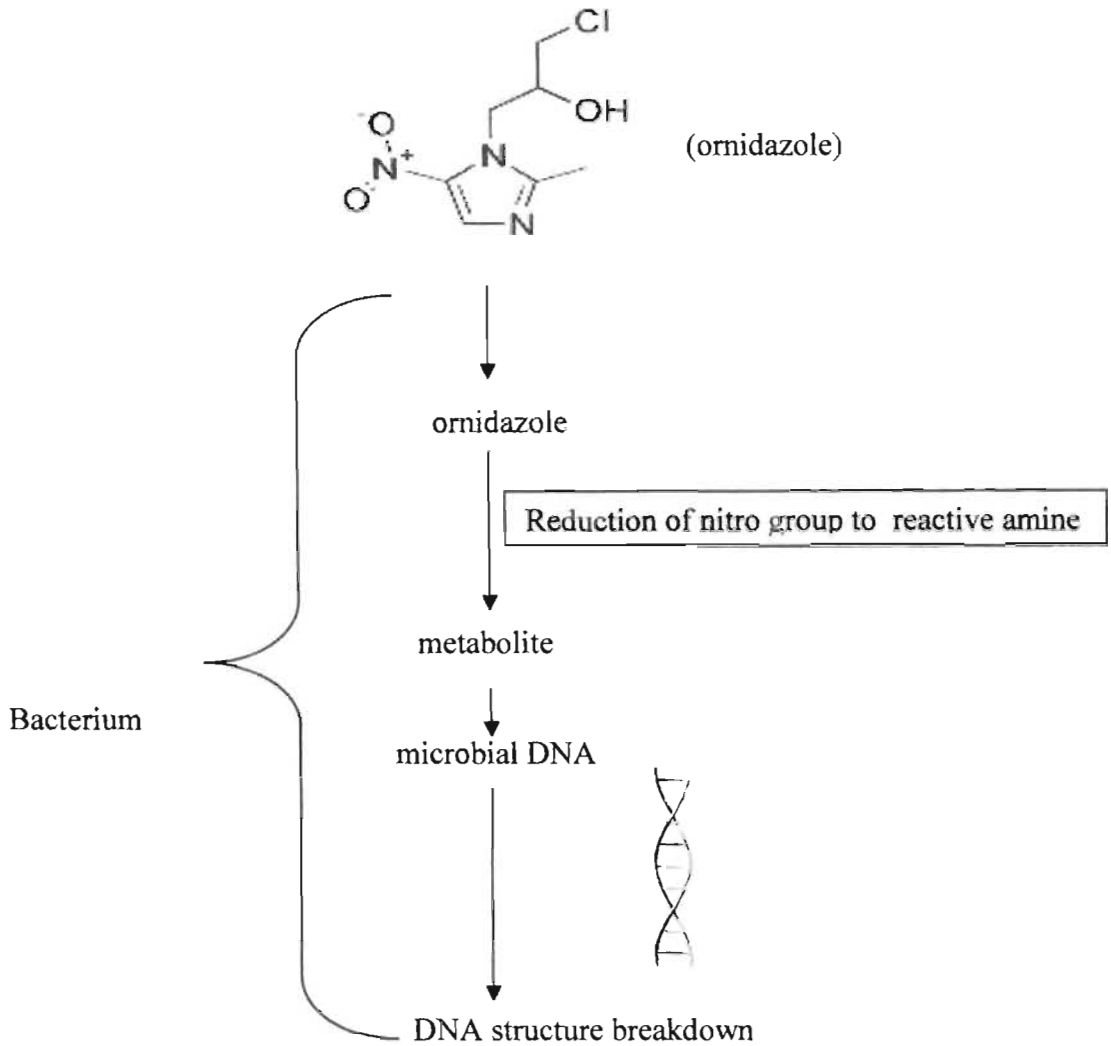

Figure 14: Mechanism of ornidazole. (ghraham G ,1997)

1.27.3 Bioavailability

1.27.3.1 Absorption

Readily absorbed (oral and intravaginal); peak plasma concentrations after 2 hr (oral), 12 hr (intravaginal). Ornidazole is almost completely absorbed from the small intestine when administered orally, with bioavailability of >90% and tmax ranging between 2 and 4 hours. Food does not affect extent but does affect rate of absorption of Ornidazole (Krishnainaya K H, 1982).

1.27.3.2 Distribution

Body tissues and fluids (wide), Protein-binding: <15%. Ornidazole is widely distributed in body tissues and fluids, including cerebrospinal fluid. Antibacterial concentrations are achieved in vaginal secreations, appendix and intestinal tissues. Ornidazole concentrations have been measured in the colonic (8.7 μg/g) and abdominal (3.6 to 4.4 μg/g) walls and epiploic fat (3.4 to 4.7 μg/g) throughout colorectal surgery in those receiving a 1 g intravenous dose for surgical prophylaxis9. Although ornidazole concentrations in cerebrospinal fluid have only been assessed in animal models, it is expected that it should penetrate the central nervous system (Krishnainaya K H,1982).

1.27.3.3 Metabolism

Ornidazole is extensively metabolized in the liver before excretion by renal pathway. Ornidazole is largely excreted in the urine and to a lesser extent in the feces, mainly as conjugates and metabolites. Only 4% of unchanged drug was excreted in the urine. 85% of a single oral dose has been reported to be eliminated within five days, 63% in the urine and 22% in the feces. Biliary excretion may be important in the elimination of Ornidazole and its metabolites3,12. Ornidazole is metabolized to five metabolites. Two of the major metabolites are M1 and M4, with M1 stemming from an oxidative pathway and M4 via hydrolysis . The following metabolites wereidentified: M1, 1-chloro-3-(2-hydroxymethyl-5-nitro- 1-imidazolyl)-2-propanol; M2, 2-methyl-5-nitroimidazole; M3, N-(3-chloro-2-hydroxypropyl) acetamide; M4, 3-(-2-methyl-5-nitro-1-imidazolyl)-1, 2-propanediol; M5, acetamide (Krishnainaya K H,1982).

1.27.3.4 Elemination

Hepatic excretion occurs via urine (as conjugates and metabolites), via faeces (small amounts); 12-14 hr (elimination half-life). The plasma elimination half-life of Ornidazole is 11 to 14 hours3,12. Plasma protein binding is approximately 11 to 13%. Reported Vss values range from 0.73 to 0.90 L/kg, with AUC values for single intravenous 500 mg doses of 185 mg/L.h and for 1 g doses of 375 mg/L.h. The mean Cl value of ornidazole is 47 mL/min (2.82 L/h) for 1 g intravenous dose . Due to its extensive metabolism, the elimination of Ornidazole is impaired in patients with severe liver disease (Krishnainaya K H, 1982).

Aim of the Study

In the perfect world amoebiasis would be prevented eradicating fecal contamination of food and water. However, providing safe food and water for the children in developing countries like Bangladesh would require massive societal changes and monetary investments. There are so many researches have been carrying out to find the efficacy of herbal product and synthetic product to treat this disease and most of the sources showed anti-amoebic properties satisfactory. Among the other drugs imidazole group shows good activity against *E. histolytica*. In this group after Metronidazole , Ornidazole comes . Other drugs are also present on this group. But their mechanism of action is not well understood. Among the two drugs on Metronidazole huge studies have done and lots of papers also published than Ornidazole. That is why this drug was selected to know it's efficacy. Several brands are also available of Ornidazole in Bangladesh. It is less costly.Therefore, the present study was aimed to find out the in vitro sensitivity of different brands of Ornidazole tablet from Bangladeshi pharmaceutical companies against clinical isolates *E. histolytica.*

Significance of the study

About 90 percent of infections are asymptomatic and the remaining 10 percent produces a spectrum varying from dysentery to amoebic liver abscess. In Bangladesh there are some pharmaceutical companies making the antiamoebics. The effects and the side effects of those drugs are known to be costly for the people who are living under poverty line in our country. Some of the synthetic drugs have lower efficacy, which can results the patients uncured or resistant to the drug forever. Indiscriminate use of drugs and different efficacy of a single drug treated with a patient at different times may lead to the physiological problems. There are so many drugs are available as antiamoebic drug in market. Ornidazole is a potential antiamoebic drug obtained from synthetic source. From some study it is observed that by using this drug the incidence of amoebic dysentery reduces effectively. It is found that the drug is safer and less toxic and efficacious than other drugs in many ways . It is a hypothesis that different brands of Ornidazole tablets from pharmaceutical companies of Bangladesh will be equally effective against the clinically isolated *Entamoeba histolytica*. This research have been carried out to know the efficacy of the different brands of Ornidazole in Bangladesh.

CHAPTER-2

MATERIALS AND METHODS

2.1 Research Design

The research design is the "*In vitro* Sensitivity of Nitazoxanide against clinical isolation of *Entamoeba histolytica*".

2.2 Collection of clinical isolation

Clinical isolates from patient attending the Out Patient Department of ICDDRB, hospital, attached to the parasitological Laboratory, ICDDRB, Dhaka, Bangladesh. Clinical isolation of *E.histolytica* were harvested from 24 h old cultures and suspended in a LYI-S-2 medium. Identification no of the clinical isolation of *E.histolytica* was 2759071. Axenic medium consists of liver digest, yeast extract, iron and serum. The parasite count was adjusted to 1.16×10^{6} mL^{-1} and 1.92×10^{6} mL^{-1}(Mukhopadhyay et.al, 1996). Isolation is usually achieved by growing the species in an environment that was previously sterilized and was thereby rid of contaminating organisms. The instruments are:

- ✓ Analytical Balance
- ✓ Morter and pastle
- ✓ Vortex machine
- ✓ Micropipettes
- ✓ Eppendrof (1ml and 2ml)
- ✓ Microtiter plate
- ✓ Microscope
- ✓ Haemocytometer
- ✓ Microtips.
- ✓ Elisa plate
- ✓ Incubator
- ✓ Autoclave
- ✓ Laminar flow
- ✓ Beaker

Figure 15: Autoclave (EWU laboratory)

Figure 16: Laminar flow(EWU laboratory)

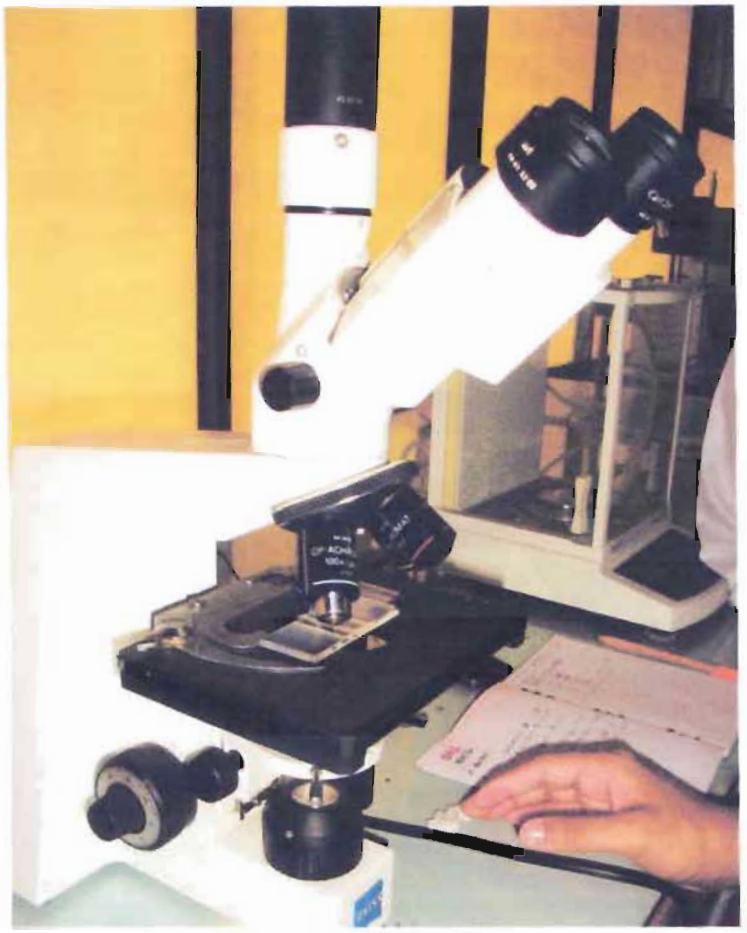

Figure 17: Microscope (EWU laboratory)

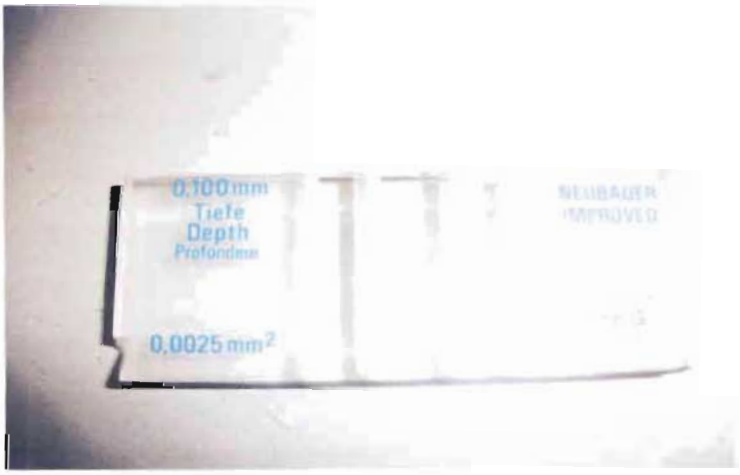

Figure 18: Hemocytometer (EWU laboratory)

Figure 19: Incubator (EWU laboratory)

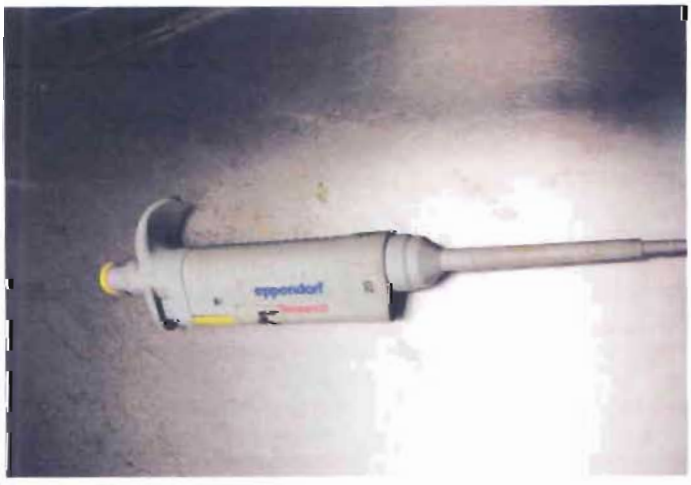

Figure 20: Micropipette (EWU laboratory)

Figure 21: Tip box (EWU laboratory)

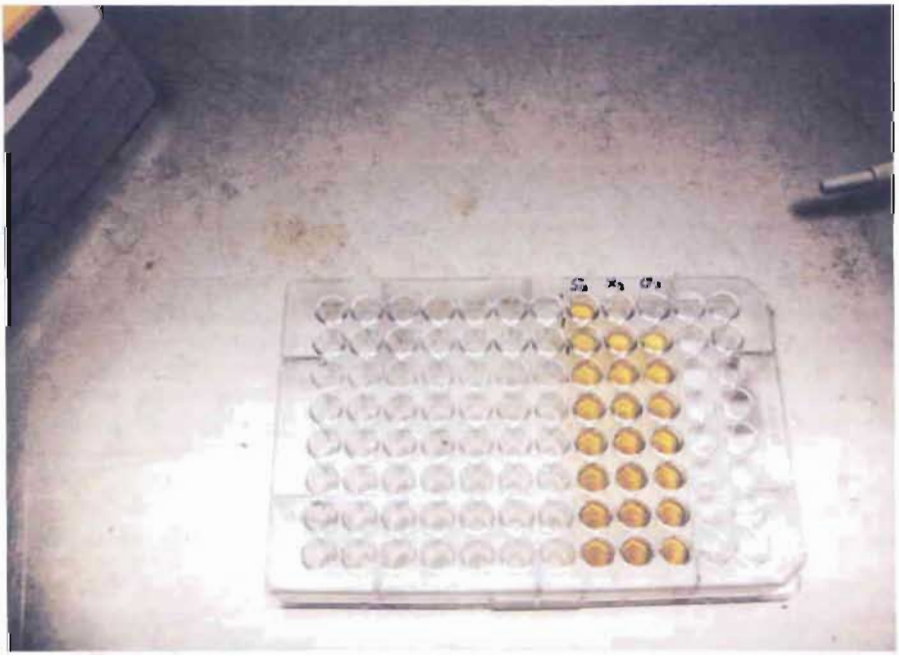

Figure 21: ELISA plate (EWU laboratory)

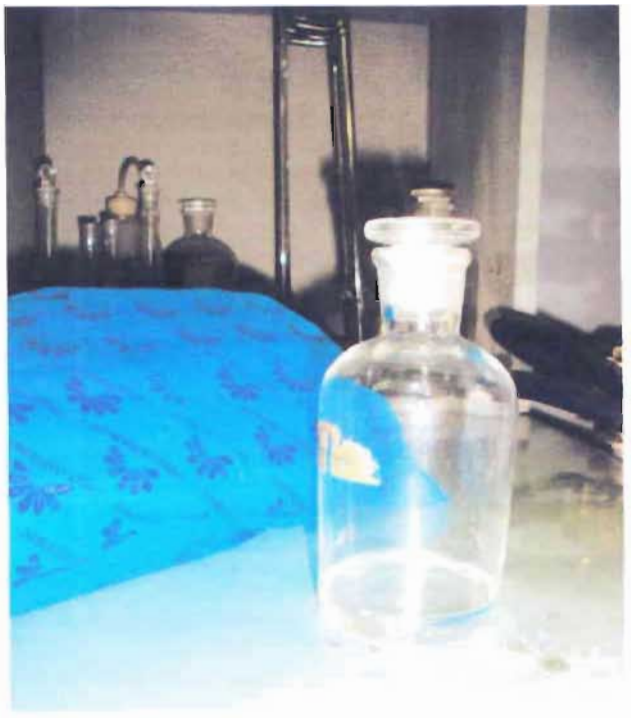

Figure 22: Ethanol & cotton(EWU laboratory)

2.3 Cultivation of *Entamoeha histolytica*

2.3.1 General Considerations

Three types of culture system will be discussed in this manual for the cultivation of *Entamoeba* species. xenic cultivation, where the parasite is grown in the presence of an undefined flora. Monoxenic cultivation, where the parasite is grown in the presence of a single species of associate and in the axenic cultivation where the parasite is grown in the absence of any other metabolizing cells. The term polyxenic is sometimes erroneously used as a synonym for xenic, however polyxenic should refer only to cultures where the identities of all the species present are known These parasitic protista are all isolated from sources rich in bacteria and fungi. Controlling or eliminating the latters growth is crucial to success in cultivating the parasites of interest. This is true even in xenic culture, where a balance between the needs of the bacterial flora and the eukaryote is important. (Dougherty et.al, 1959) .Unlike *Trichomonas vaginalis* and *Giardia intestinalis* which can readily be established directly into axenic cultures, *Entamoeba histolytica* has never been grown axenically without first being established in culture

with other organisms and usually with a complex undefined bacterial flora. Axenization of *E. histolytica* is a laborious and lengthy process where xenic cultures of the organism are first adapted to monoxenic growth, usually with a kinetoplastid flagellate as an associate, before weaning them from a phagocytotic lifestyle to one where the nutrients are obtained largely by pinocytosis (Diamond L S, 1968).

2.3.2 Historical background

Entamoeba histolytica was first established in culture by Boeck and Drbohlav in 1925 in a diphasic egg slant medium they had developed for isolation of intestinal flagellates .(Boeck et.al,1925) A modification of this medium (LE) is still in use today. Their success refuted the Promethean view of *E. histolytica* as an obligate tissue parasite. Dobell (Dobell C et.al,1926) introduced the use of rice starch as a carbohydrate source, and it remains a component of all media for xenic cultivation to this day. Soluble sugars, were they to be used, would be metabolized too rapidly by the bacteria and would not be available to the amoebae. Several diphasic media were developed subsequently with serum, agar or egg extracts in the slants. (Diamond, L. S. 1982) Currently the most widely used media for xenic cultivation of *E. histolytica* are the diphasic LE and Robinson's (Robinson, G. L. 1968) media and the monophasic TYSGM-9. (Diamond, L. S. 1983) .Monoxenic cultivation of *E. histolytica* was first accomplished by Cleveland and Sanders (Cleveland et.al,1930) in a diphasic medium with a single species of bacterium, and the monobacterial Modified Shaffer-Frye medium (MS-F) was widely used at one time. (Reeves et.al, 1957) Monoxenic cultivation is of limited use today except as a transitional stage between xenic and axenic cultures. *Crithidia fasciculata* or *Trypanosoma cruzi* are the associates of choice for monoxenic cultivation. (Diamond L S, 1968)

2.4 Media for Axenic cultivation

Axenic cultivation of *E. histolytica* was first accomplished by Diamond in 1961 (Diamond, L. S. 1961). The medium used was complex diphasic serum-enriched nutrient agar slant overlayed with a broth supplemented with chick embryo extract and vitamins. It was not until Diamond introduced the monophasic medium TP-S-1 in 1968 that axenic cultures of *E. histolytica* started to be widely used. TP-S-1 was superseded by TYI-S-33.

Diamond et.al,1968)and this is currently the most widely used medium for axenic ultivation of *E. histolytica*. (Gillin et.al 1978)

.5 Media for Xenic Cultivation

Xenic culture will be for identification of the species of *Entamoeba* present in the ample, for example by isoenzyme analysis. Several intestinal species of *Entamoeba* can e found in human who are sufficiently similar to cause diagnostic confusion and all can row in the same media. Because *E.histolytica* is the only species that cause invasive isease, differentiation from the closely related, more common, and morphologically dentical species *E. dispar* in particular is desirable, in order to prevent unnecessary hemoprophylaxis. Until less laborious method become widely tested and implemented soenzysme analysis will remain the standard for separation of these two parasites.The nost common source of material will be stool samples. In rare instances rectal biopsies r liver abscess aspirates have been the starting point for cultures .In the latter case since he abscess is sterile, addition of a bacterial flora is used for the direct establishment of nonixenic cultures. Unless the stool sample is several approaches to the establishment of ultures. Procedure for it first large beaker was taken then 1 lit distilled water was taken 7 gm of sodium chloride was added then heat and 15 gm of Bacto TM agar media was dded. Then again heat after the heat the solution must be cool and taken into a small lass bottle by a syringe .Then placed in the auto clave, after completed the auto clave, he liquid solution bottle taken into a box and the box must be settled by an angle state ecause the angle bottle can be suitably for parasite. (Jean-Francois et.al, 2010)

2.6 Preparation of Rice Starch

Purified rice starch is important for all these media. To prepare (Diamond, L. S. 1983), placed 500 mg of powdered rice starch into each of several 16x125mm culture tubes and heated at 150 ° C, with loose caps, in a dry oven for 2.5 hours with the starch distributed along the length of the horizontal tubes. Sterilization of the rice starch prevents alteration of the bacterial flora when it is added to the culture and is thus recommended. After cooling, tightened the caps and stored at room temperature. To prepare for use, added 9.5 ml of sterile distilled water or phosphate buffered saline (PBS) to one tube and vortex to resuspend. Distributed the 1 ml of the resuspended starch to each of 10 tubes containing 9 ml of sterile water or PBS and refrigerate. The final concentration of diluted rice starch is 5 mg/ml. Before use, resuspended the rice by vortexing or vigorous shaking and

pipeted the desired volume into culture tubes with medium making sure that the stock rice stays in suspension. Different isolates require varying amounts of rice starch but 0.2 ml is often a suitable amount to add per culture tube. *Entamoeba* will not ingest all forms of rice .Most important is the size of the rice particle as it must be within the ameba's ability to phagocytes it. (Diamond, L. S. 1983)

2.7 Diphasic Media

2.7.1 Locke-egg (LE) medium

Prepare Locke's solution: To prepare the Locke's solution dissolve the followings materials:

- 8.0 g Sodium Chloride
- 0.2 g Calcium Chloride
- 0.2 g Potassium Chloride
- 0.01 g Magnesium Chloride
- 2.0 g Sodium Phosphate, dibasic

Then autoclave it for 15 minutes at121° C and at 15 lbs. pressure. It must be cooled to room temperature and removed any precipitate by filtration .And then re-autoclaved to sterilize. (Von Brand et.al 1943)

2.7.2 Preparation of egg slant

To prepare the egg slant the first procedure is surface sterilize fresh hens eggs by flaming in 70% ethanol and break into a graduated cylinder. Then add 12.5 ml Locke's solution per 45 ml of egg. After this emulsify in a Waring-type blender and filter through gauze into a flask. Then it must be placed under the vacuum to draw out all air bubbles.Then the second stage is the addition of 5 ml amounts of the emulsified egg to standard 16x125 mm culture tubes and autoclave at 100° C for 10 minutes. The resulting egg slants should be free of bubbles. Cool to room temperature and overlay slants with 6 ml Locke's solution and autoclave for 15 minutes at 121° C and at 15 lbs. pressure. After cooling to room temperature tighten the caps and refrigerate for up to 6 months (Von Brand et.al 1943).

2.7.3 Preparation of Robinson's medium

This is a complex medium that has nevertheless found widespread use for the isolation of enteric amoebae. It is prepare by the following 06 stock solutions. Stock solutions are:

1. **0.5% Erythromycin:** 0.5% Erythromycin is prepared in distilled water and filter then sterilize and finally Refrigerate.
2. **20% Bactopeptone:** 20% Bactopeptone is prepared in distilled water. Autoclave the solution and refrigerate.
3. **10X Phthalate solution stock:**

It is prepared by mixing following ingredients:

- 102 g Potassium Hydrogen Phthalate
- 50 ml of 40% Sodium Hydroxide

Prepared to 1 liter stock solution, and then adjusted the pH at 6.3. Then the solution is autoclaved for 15 minutes at 121° C and at 15 lbs. pressure. Then the solution was stored at room temperature. It is diluted 1:10 with sterile water before use. A stock of Phthalate can be made by adding 1.25 ml of 20% Bactopeptone per 100 ml 1X Phthalate solution. Then the solution is refrigerated for the storage.

4. **10X R Medium stock:** It is prepared by dissolving the following ingredients in distilled water:

- 25.0 g Sodium Chloride
- 10.0 g Citric Acid
- 2.5 g Potassium Phosphate, monobasic
- 5.0 g Ammonium Sulfate
- 0.25 g Magnesium Sulfate.7H2O
- 20 ml 85% Lactic Acid solution.

Then prepared to 500 ml stock solution diluted stock 1:10 adjusting the pH to 7.0 and autoclaved it for 15 minutes, at 121° C, and at15 lbs. pressure in 20 ml amounts.

5. **BR medium:** To prepare the BR medium inoculated 1X R medium with a standard *Escherichia coli* strain .Incubated it at 37° C for 48 hours and stored at room temperature.

6. **BRS medium:** To prepare the BRS medium added an equal volume of heat inactivated bovine serum to BR medium and incubate at 37° C for 24 hours. Then stored at room temperature.

.7.4 Preparation of agar slants

To prepare the agar slants use 1/2 oz.Quorpak bottles. Autoclave a solution of 1.5% Noble Agar in 0.7% Sodium Chloride water for 15 minutes at 121° C with 15 lbs. pressure. Dispense in 5 ml or 7 ml amounts, re-autoclave and slant until cool and set. When cool, tighten lids and store at room temperature or refrigerated.To one tube or bottle add the following : 3 ml 1X Phthalate Bacto peptone, 1 ml BRS, 50 µl Erythromycin. This must be done shortly before inoculation

2.8 Monophasic culture media

2.8.1 TYSGM-9

TYSGM-9 it is one kind of mono phasic media which is prepared by dissolving the following

compounds. The compounds are:

- 2.8 g Potassium Phosphate, dibasic
- 0.4 g Potassium Phosphate, monobasic
- 7.5 g Sodium Chloride
- 2.0 g Casein Digest Peptone
- 1.0 g Yeast Extract

To prepare the 950 ml solution with distilled water. Dispense it in 95 ml amounts and add 0.2 g bovine gastric mucin to each bottle. Then it autoclave for 15 minutes at 121° C with 15 lbs. pressure and store in the refrigerator. Before use, add 0.1 ml of a filter sterilized 5% stock of Tween 80 in distilled water and 5 ml of heat inactivated adult bovine serum. At last dispense in 8 ml amounts into 16x125mm culture tubes. (Diamond et.al, 1982)

.8.2 LYSGM

YSGM is a modification of TYSGM-9 that removes the need for Casein Digest eptone and it is replaced by 0.5g of neutralized liver digest and the amount of Yeast xtract is increased to 2.5g. It is derived from LYI-S-2 in the same way that TYSGM-9 s derived from TYI-S-3.

.9 Axenic Culture Media

One constant problem facing those who rely on axenic cultures is the fastidiousness of hese organisms. Although the others are also affected to a significant degree, this is specially true of *E. histolytica*. Lot to lot variations in several components of the axenic ulture media in particular can have profound effects on the ability of a medium to upport growth of the organisms, some lots may even be toxic. Trypticase, yeast extract, nd serum are the medium components most commonly affected, but the quality of the listilled water and even the type of glass used in making the culture tubes can cause. For his reason, we highly recommend that those wishing to undertake axenic cultivation of hese organisms test the ability of each new lot of reagent to support growth before tarting to use it. (Diamond, L. S et.al, 1991)

2.9.1 Procedure for the preparation of Axenic culture media for E. histolytica

(i) TYI-S-33

To prepare TYI-S-33 dissolve the following in this order in 600 ml of deionized or glass-distilled water, 1.0 g of potassium phosphate, dibasic, 0.6 g of potassium phosphate, monobasic, 2.0 g of sodium chloride, 20.0 g of casein digest peptone, 10.0 g of yeast extract, 10.0 g of glucose, 1.0 g of L-cysteine hydrochloride, 0.2 g of ascorbic acid, and 1.0 ml of ferric ammonium citrate. (*Diamond*, L. S et.al 1978). The final volume was brought to 880 ml and pH to 6.8 using 1 N sodium hydroxide solution. Dispense in 88ml amounts into 125ml glass bottles and autoclave for 15 min at 121°C under a pressure of 15 lbs. Then sterile TYI base can be stored frozen at 20°C for several months. Vitamin mix 18 unlike earlier mixtures used in the axenic culture of *E. histolytica*, contains only those vitamins known to be required by the parasite. It is also available commercially. (Diamond, L. S et.al, 1991)

(a) ***Step 1.*** The following four solutions must be prepared and then combine. (i) Dissolve 45 mg of niacinamide, 4 mg of pyridoxal hydrochloride, 23 mg of calcium pantothenate,

5 mg of thiamine hydrochloride, and 1.2 mg of vitamin B_{12} in a final volume of 25 ml of water. (ii) Dissolve 7 mg of riboflavin in water using the minimum amount of 0.1 N sodium hydroxide, bringing the final volume to 45 ml. (iii) Dissolve 5.5 mg of folic acid in water using the minimum amount of 0.1 N sodium hydroxide, bringing the final volume to 45 ml. (iv) Dissolve 2 mg of D-biotin in water bringing the final volume to 45 ml. If the combined solution is cloudy, it indicates that the pH is too high due to too much sodium hydroxide having been used in solutions ii and iii and the mixture must be discarded. (*Kobayashi*, S et.al,1998)

(b) ***Step 2.*** In step 2 dissolved 1 mg of DL-6-8-thioctic acid in 5 ml of 95% ethanol. Add 500 mg of Tween 80, bringing the volume to 30 ml with water. Combine the solutions from steps 1 and 2, bring the final volume to 200 ml with distilled water, and sterilize through a 0.22μm pore size filter. Store in 100 ml amounts at 4°C for up to 6 months. To complete TYI-S-33 medium for use in axenic cultivation add 2.0 ml of vitamin mix 18 and 10 to 15 ml of heat-inactivated adult bovine serum to each 88 ml of TYI broth. In most cases 13 ml of TYI-S-33 per tube is the correct amount, as inocula for axenic *E. histolytica* are generally small in established cultures. The percentage of serum used varies among isolates but is usually either 10 or 15 %.(Zierdt, C. H et.al, 1974)

(ii) YI-S. YI-S was developed as an alternative to TYI-S-33 due to difficulties in obtaining lots of casein digest peptone that would support adequate growth of *E. histolytica*. The recipe for YI-S is identical to that of TYI-S-33 except that casein digest peptone is replaced weight for weight by additional yeast extract, making the final concentration of yeast extract 3%. YI-S is not without its own problems, as the lot of yeast extract used is crucial to successful cultivation using this medium.(Diamond, L. S et.al,1995)

(iii) LYI-S-2. In the course of developing YI-S, several combinations of liver digest and yeast extract are used. One of these, designated LYI-S-2, it is found to result in growth equal to that in TYI-S-33. Intent on producing a medium with as few biological ingredients as possible, the medium containing only yeast extract that is YI-S. It has been used in the long-term cultivation of several isolates of *E. histolytica* and a number of other *Entamoeba* species. (Ho, L. C et al 1993)

The quantities composition of LYI-S-2 is given below:

Table-7 The quantities composition of LYI-S-2

Ingredients	Amount
Potassium phosphate dibasic	1.0 gm
Potassium phosphate monobasic	0.6 gm
Sodium chloride	2.0 gm
Yeast extract	25.0 gm
Liver extract	5.0 gm
Glucose	10.0 gm
Cysteine	1.0 gm
Water (distilled / deionized)	880.0 mL
Bovine serum	15.0 %
Vitamin mix	2.0 %

2.10 Establishment of cultures

It is very important to remember that a negative culture does not mean that the patient is uninfected. None of the organism being considered here produce cultures 100% of the time from microscopically the positive samples, and in the case of *E.histolytica* the success rate appear to be between 50 and 70 % in most laboratories, based on personal communication. It is also important to remember that what grows in culture is not necessarily the organisms are seen by microscopy.

2.11 General Considerations for the preparation of Xenic culture

Usually, the reason for establishing xenic cultures will be for identification of the species of *Entamoeba* present in the sample, for example by isoenzyme analysis. Several intestinal species of *Entamoeba* can be found in humans who are sufficiently similar to cause diagnostic confusion and all can grow in the same media. Because *E. histolytica* is the only species that causes invasive disease, differentiation from the closely related, more common, and morphologically identical species *E. dispar* in particular is desirable, in order to prevent unnecessary chemoprophylaxis. Until less laborious methods become widely tested and implemented, isoenzyme analysis will remain the standard for the separation of these two parasites.The most common source of material will be stool samples and this is what is assumed below. In rare instances rectal biopsies or liver

abscess aspirates have been the starting point for cultures. In the latter case, since the abscess is sterile, addition of a bacterial flora is necessary before inoculation of amoebae into xenic culture. Such material has also been used for the direct establishment of monoxenic cultures. Unless the stool sample is from a patient with dysentery the amoebae will be in the encysted form. This allows for several approaches to the establishment of cultures.(Dobell,C et.al 1926)

2.12 Elimination of unwanted organisms

One of the banes of xenic cultivation is the likelihood of unwanted organisms outgrowing the desired ameba. The most frequent source of this problem is *Blastocystis hominis* which may be the most common parasitic infection of humans. This organism is often missed on stool examination but grows luxuriantly in all the media used to cultivate xenic *Entamoeba.* Some authors control the growth of *B. hominis* with acriflavin but this also has an adverse effect on the bacterial flora and, directly or indirectly, on the ameba of interest. We have successfully used two methods to eliminate *B. hominis* from *Entamoeba* cultures.In first method, cysts are treated with 0.1N hydrochloric acid at room temperature for 10 minutes, washed thoroughly with distilled water, and re-inoculated into culture medium to which a suitable bacterial flora has been added. The acid kills the bacteria, any fungi *B. hominis*, intestinal trichomonads and any non encysted amoebae while leaving the cysts intact and viable. The bacterial flora used in the above method is separated from another xenic culture by inoculating into culture medium, without rice starch, a small amount of supernatant from an established culture, subculturing twice and refrigerating the flora for 48 hours. The successful separation of the flora can be checked by inoculating a substantial volume into fresh medium with rice starch and checking for amebas growth. The flora can be stored at 4° C indefinitely. (Dobell, C et.al 1926).The second method is called "Smedley method" and is used when *B. hominis* appears in cultures. It does not rely on cysts being present and so has advantages over the method of Dobell and Laidlaw in that respect. However, the method may need to be repeated a couple of times before the *B. hominis* is completely eliminated. Cultures are pelleted and the pellet, which contains a mixture of all the organisms present, is resuspended in distilled water at room temperature for 15 minutes. The material is then re pelleted and inoculated into fresh culture medium. Perhaps surprisingly, many *Entamoeba* trophozoites survive this treatment while *B. hominis*

generally does not. A few cells of *B. hominis* may survive and start to grow, and the procedure will then need to be repeated. The advantage of Smedley's method is its simplicity and the fact that no separate bacterial flora is needed. Other unwanted organisms such as fungi and trichomonads will usually disappear from xenic cultures after several passages. (Smedley, S. R. 1956)

2.13 Isolation

In our experience LE has proven to be the best medium for primary isolation of *Entamoeba* species from stool, although we have limited experience with Robinson's medium which is widely used for this purpose. TYSGM-9 can also be used for isolation but its primary utility is in generating large numbers of amoebae from established cultures. The numbers of amoebae obtained from the two diphasic media are generally low in comparison with TYSGM-9, but their success in primary isolation amoebae from microscopically positive stool is higher. In all cases, rice starch is added to the medium before inoculation, as are the antibiotics when needed. Material for inoculation of xenic cultures can be prepared in several ways. Most commonly, stool samples are emulsified in saline and passed through a mesh to remove most of the larger particulates from the material before addition to the culture medium. It is always a good idea to include portions of the stool that appear bloody if these are present. Stool fractionation by flotation in zinc sulfate is also used as this reduces the amount of debris while concentrating the cysts present in the sample. We routinely use more than one medium, if available, and set up duplicate cultures where one has antibiotics added and the other not. Penicillin/streptomycin or erythromycin is the antibiotics of choice as they appear to have little direct effect on the amoebae. However, the widespread occurrence of antibiotic resistance in bacteria makes it impossible to generalize about the amount and type of antibiotics necessary to control the growth and rice-splitting activity of human bacterial flora. Culture tubes, containing medium and rice starch, to which stool derived material has been added are incubated vertically at 35.5° C for 48 hours before examination. Ideally, examination should be done in situ if possible. This can be accomplished by slanting the tubes and using an inverted microscope. Amoebae can be observed adhering to the walls of the glass culture tubes above the fecal material. In situ examination is much easier in monophasic medium due to its relative . The culture tube is chilled in an ice water bath for 5 minutes and most of the liquid overlay discarded to

leave less than 1 ml in the tube. The sediment is resuspended in the remaining fluid and transferred to a fresh culture tube with medium and rice. After incubation for a further 48 hours the culture is re examined as above. If no amoebae are seen the subculture method is identical except that the size of the inoculum will be gradually reduced as the numbers of amoebae increase. Initially, it is usually helpful to centrifuge the cultures and split the pellet among the recipient tubes. This can be done by chilling the culture tube, inverting several times to detach the adherent amoebae, and transferring the liquid phase to an empty culture tube before pelleting. (Diamond, L. S. 1968). Alternatively, a drop of sediment can be extracted from the tube for examination on a microscope slide. If no growth is observed at 48 hours a blind passage should be made.

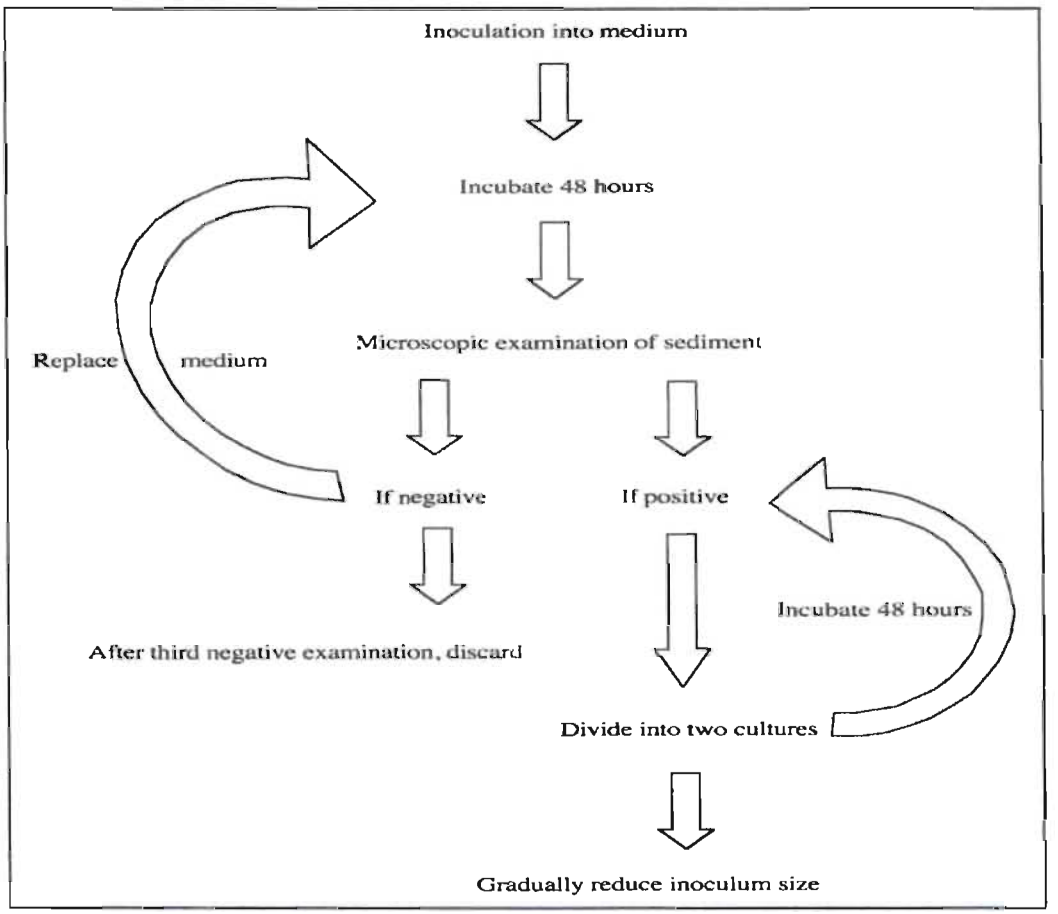

Figure 24: flow diagrams illustrating the stages in establishing luminal protists in culture.

2.14 Axenization

Axenization of *E. histolytica* is a long and laborious procedure involving gradual adaptation of the parasite to a new way of life. The medium can be a specialized monoxenic culture medium but we have also use success initiating such cultures using one of the axenic media such as TYI-S-33. The monoxenic associate we have used most frequently is *C. fasciculata* .This insect flagellate is grown as a stock culture at room temperature and added to the monoxenic culture of amoebae at each subculture, as *Crithidia* does not grow at the incubation temperature of the amoebae; the amount added varies. *T. cruzi* Culbertson has also been used successfully as the associated organism but is not recommended due to the potential for infection, even though this strain is of very low virulence. The antibiotics added vary both in type and amount depending on the sensitivities of the flora in which the amoebae were growing. We have used a cocktail of rifampin, amikacin, oxytetracycline, and cefotaxime with good success. Except for the first agent, these have little effect on the amoebae. The initial concentration is often as high as 0.1 mg/ml of culture medium. After 24 h, the cells are pelleted by centrifugation and the medium is replaced. As the ameba cell numbers increase, the cell pellet can be divided between two tubes. By reducing the antibiotic concentration gradually in one of a pair of tubes to test for bacterial growth, sterility can be achieved gradually while at the same time the numbers of amoebae are increasing (Diamond, L. S. 1968).At least two subcultures in the absence of antibiotics should be performed before the cultures can be considered free of bacteria. This can be verified using standard aerobic and anaerobic testing procedures for bacteria, including mycoplasmas and fungi. Established monoxenic cultures, those in which growth is reproducible and bacteria are absent, are then used to initiate axenic cultures. This uses the same medium but with no *Crithidia* added. After a few subcultures the flagellates disappear as a result of dilution and ingestion. It is often helpful, although not always necessary, to add a small amount of Noble agar to the tubes .It appears to form a substrate for the amoebae. In addition, the tubes should be incubated vertically rather than at 5° to the horizontal. Often the culture will flourish initially and then numbers will crash. It is the crisis point that the cultures are most vulnerable. As long as a few live cells persist, it is worth continuing to replace the medium every few days. With luck, the numbers will gradually start to increase again, and eventually addition of the agar will no longer be needed. When established, the axenic cultures can be incubated at 5° to the horizontal (Diamond, L. S. 1983)

2.15 Principles of inducing encystment of *E. histolytica*

The methods of inducing encystment of *E. histolytica* are based on Dobell and Laidlaw's discovery that cyst-production may sometimes be temporarily increased by cultivating the amoebae in starch-free media for one or two generations, and then transferring them to media containing this substance but the results are uncertain, and the number of cysts produced in any culture cannot be predicted. Here at present we use a protocol that is used for many years in the NIH Laboratory of Parasitic Diseases. Three things are of special concern in obtaining cysts they are the media, bacterial flora, and rice starch. Some media are better than others for this purpose. LE medium is the one with which we and others have had excellent results The accompanying bacterial flora present in a xenic culture plays an important role in the process of encystment. It is good practice for those requiring a steady source of large numbers of cysts to isolate and maintain the bacterial flora of a xenic culture in which cysts regularly form spontaneously (Dobell, C et.al, 1926).

2.15.1 Protocol of encystment

(a) Day 1.

At first the process is begin with three amoebae rich 48 h cultures in LE medium. Harvest them by chilling the culture tubes for 5 min in an ice water bath then invert the tubes 10 times to mix contents and free amoebae adhering to the glass and egg slant, and centrifuge for 3 min at 275 x *g*. Remove and discard all but 1 ml of the spent overlay. Finally resuspend pelleted amoebae, pool, and transfer equal amounts to six tubes of LE medium without rice. Incubate the cultures in an upright position for 72 h.

(b) Day 4.

Harvest each of the six cultures: Chill, remove and discard all but 1 ml of overlay. Mix remaining overlay of each culture and transfer equal amounts to two tubes of LE medium without rice. There will now be 12 cultures. Then it is incubate for 48 h.

(c) Day 6.

Harvest the 12 cultures and subculture as on day 4. Then the 24 cultures must be incubate for 48 h.

(d) Day 8.

In day 8 carefully remove the overlay from each culture, leaving only enough to cover the sediment at the interface of the egg slant and overlay. The sediments must be collected from three cultures and transfer to one tube of medium to which rice has been added. Repeat with the remaining cultures. Incubate the resulting eight cultures for 48 h.

(e) Day 10.

Remove a small drop of sediment from each culture, stain with Lugol's iodine solution, and search for presence of quadrinucleate cysts. If found, harvest cultures as on day 1. Remove overlay, leaving only the sediment. Pool sediments and wash two times with distilled water. Cysts will remain viable from 10 to 14 days when stored at 4°C. If cysts are not found, then incubate it with additional 24 h.

2.15.2 Protocol of excystment of cysts induced in vitro

Inducing *E. histolytica* to excyst is relatively easy compared to getting the ameba to encyst. If the goal is to propagate the amoebae in a xenic environment, then the medium in which the cysts were induced is used, in this case LE medium. If the goal is to excyst them in a bacteria-free environment, any of the monophasic liquid media devised for axenic culture can be used. In the latter case freshly prepared medium must be used. Best results are obtained when at least 50% of the cysts produced are in the quadrinucleate stage. Usually no more than 25% of the cysts can be expected to excyst. To induce excystment, the cysts are first treated to remove unwanted organisms. They are then placed in a tube of LE medium inoculated with a suitable bacterial flora for xenic growth or in a medium capable of sustaining axenic growth. Upon incubation most of the cysts capable of undergoing excystation .

2.16 Rexenization of axenically cultivated *E. histolytica*

Occasions will arise when it is desirable to return agenized amoebae to the xenic state. The following protocol has worked

(i) Inoculation of the three tubes of LE medium with a bacterial flora known to support xenic growth.

(ii) Chilling a 72-h culture of axenically cultivated amoebae in an ice-water bath for 5 min. Invert culture tube 10 times to dislodge amoebae from glass surfaces. Centrifuge 3 min at 275 x *g*. Remove supernatant and discard.

(iii) Resuspend amoebae in 1 ml of fresh medium for axenic culture, count cells, and inoculate the tubes of LE medium with 1 x 10^5, 2 x 10^5, and 4 x 10^5 amoebae, respectively.

(iv) Incubate 48 h. Remove all but approximately 1 ml of overlay. Resuspend the sediment located at the interface of the slant and overlay. Examine a drop with a microscope. The majority of inoculated amoebae will have died. Select the best of the three cultures and subculture.

(v) The number of amoebae transferred can be determined only by trial and error. In the early stages of establishing the culture, transfer one-half of the material from the old culture to each of two tubes of fresh medium. Later, as amebic growth improves, transfer smaller portions, e.g., one-third to one fourth.

2.17 Maintenance of culture

When the culture established all parasites then they are handled in the same way. Xenic cultures of *E. histolytica* is routinely passaged at 48 to 72h intervals, usually a Sunday-Tuesday schedule is convenient. Occasionally cultures of these organisms will be found that do better with twice weekly subculture. The inoculum size for the longer incubation period should be smaller than that for shorter incubations. However, variation among isolates and flora means that no generalities can be made regarding the size of inocula or the amount of rice and antibiotics to be added to the medium for optimal growth. It is very much a case of trial and error combined with experience in evaluating growth of cultures that leads to successful establishment of these parasites in xenic culture. It is recommended that xenic cultures be passaged using two or more inoculum sizes to ensure a successful subculture. A significant threshold effect can sometimes be encountered, in which a certain inoculum size gives rise to a healthy culture but an inoculum smaller by as little as 50 µl may result in no growth. An increased inoculum volume may be warranted for the subsequent subculture to compensate for the dead amoebae. The method for subculturing is essential. Cultures are chilled in an ice water bath for 5 min or

10 min to release trophozoites attached to the glass culture tube. Tubes are inverted several times to disperse the cells and a measured inoculum is passed aseptically to a culture tube that contains the fresh medium. The tubes are capped tightly and incubated at 36 to 37°C.

2.18 Preparation of Antimicrobial agent

The standard Ornidazole drug used in the study was colledted as pure salt from **UniMed Unihealth Pharmaceuticals Ltd, Dhaka, Bangladesh** . Standard Ornidazole was weighed and dissolved in 1mL of distilled water .The stock solution was stored in a refrigerator.

2.19 *In vitro* drug sensitivity assay:

Drug sensitivity assay of the sample was carried out by using microtiter plates. In row A 200 micro liter of the standard was given and the samples were given. In all other rows (B-H) the 100 micro liter medium was added and dilution of the drugs were performed down the plate then mixed properly. 100 micro liter of the medium from the last row (H) was discarded to maintain the equality of the concentration of the drugs. The final concentration of the drug were 4.6, 2.3 , 1.15, 0.575, 0.287, 0.1437 μM. Further 100 μL of parasite suspension was added to all the rows (A-H). Each test included the control where no drug is present. Then plastic strip was used to cover the plate. Plates were incubated at 37°C and examined after 1 or 2 h under a microscope to check for the presence of amoebac. After 4 h the plate was taken from the incubator. Then the viable parasites were counted by haemocytometer under microscope in each of the rows.

CHAPTER-3

REASULTS

3.1 Measurement of amoebicidal activity

The clinical isolates of *Entamoeba histolytica* was treated with different brands of Ornidazole at different concentration. The experimental concentrations were 0.144, 0.288, 0.575, 1.15, 2.3 and 4.6 µM. A control group was made to measure the change in the viable counts of *E. histolytica.* Each ELISA plate contained different concentration of Ornidazole brands and *Entamoeba histolytica* (100 micro liters). After that the plate was incubated for 24 hours. Then viable and non viable *Entamoeba histolytica* were counted and recorded.

3.2 Viable count of clinically isolates *E. histolytica* from standard & commercial product of Ornidazole (Xynor) after 24 hours of incubation

The initial count of the parasites were 1.575×10^5 mL^{-1} . After 24 hours the parasites count of the control were 3.95×10^5 mL^{-1}. The number of parasites were increased in the control after 24 hours of incubation.

Table-8 Viable count of *E. histolytica* in Standard & brand of Ornidazole (Xynor) after 24 hours of incubation.

Concentration of Ornidazole (µM) Standard	**Viable count of *E. histolytica***	**Concentration of Ornidazole (Xynor) (µM)**	**Viable count of *E. histolytica***
4.6	0.12×10^5 mL^{-1}	4.6	$0.12 \times 10^5 mL^{-1}$
2.3	$0{\cdot}15 \times 10^5 mL^{-1}$	2.3	$0{\cdot}17 \times 10^5 mL^{-1}$
1.15	0.20×10^5 mL^{-1}	1.15	0.22×10^5 mL^{-1}
0.58	$0.25 \times 10^5 mL^{-1}$	0.58	$0.42 \times 10^5 mL^{-1}$
0.29	$0.3 \times 10^5 mL^{-1}$	0.29	$0.47 \times 10^5 mL^{-1}$
0.14	$0.4 \times 10^5 mL^{-1}$	0.14	$0.82 \times 10^5 mL^{-1}$

After 24 hours incubation ,when the concentration of Ornidazole was 0·144 µM the viable count

of *Entamoeba histolytica* were $0.4\times10^5 mL^{-1}$ for standard and $0.8\times10^5 mL^{-1}$ for brand respectively. In the standard preparation , the viable count of clinically isolates *Entamoeba histolytica* were $0.3\times10^5 mL^{-1}$, $0.25\times10^5 mL^{-1}$, 0.20×10^5 mL^{-1} and $0{\cdot}15\times10^5 mL^{-1}$ when the concentrations were 0.287, 0.575, 1.15 and 2.3 μM respectively. In case of Ornidazole brand (Xynor) the viable count for clinically isolates *Entamoeba histolytica* were $0.47\times10^5 mL^{-1}$, $0.42\times10^5 mL^{-1}$, 0.22×10^5 mL^{-1} and $0.12\times10^5 mL^{-1}$ when the concentration were 0.287, 0.575, 1.15 and 2.3 μM respectively.

When the concentration of Ornidazole (standard) was increased to 4.6μM ,the viable count of clinically isolates *Entamoeba histolytica* was decreased to 0.12×10^5 mL^{-1} and for the Ornidazole brand (Xynor) ,the count of clinically isolates *Entamoeba histolytica* was decreased to 0.15×10^5 mL^{-1} .

Table-9 Percentage of viable count of *E. histolytica* from standard and commercial product (Xynor) of Ornidazole after 24 hours of incubation.

Concentration of Ornidazole (μM) Standard	**Viable count of *E. histolytica* in percentage**	**Concentration of Ornidazole (Xynor) (μM)**	**Viable count of *E. histolytica* in percentage**
4.6	7.61 %	4.6	7.93%
2.3	9.52 %	2.3	10.79%
1.15	12.69 %	1.15	14.28 %
0.58	15.87 %	0.575	26.92 %
0.29	19.04 %	0.288	30.15 %
0.14	25.39%	0.144	52.38 %

Figure-25 : Concentration of viable count of *E. histolytica* from standard & commercial product (Xynor) of Ornidazole after 24 hours of incubation in percentage

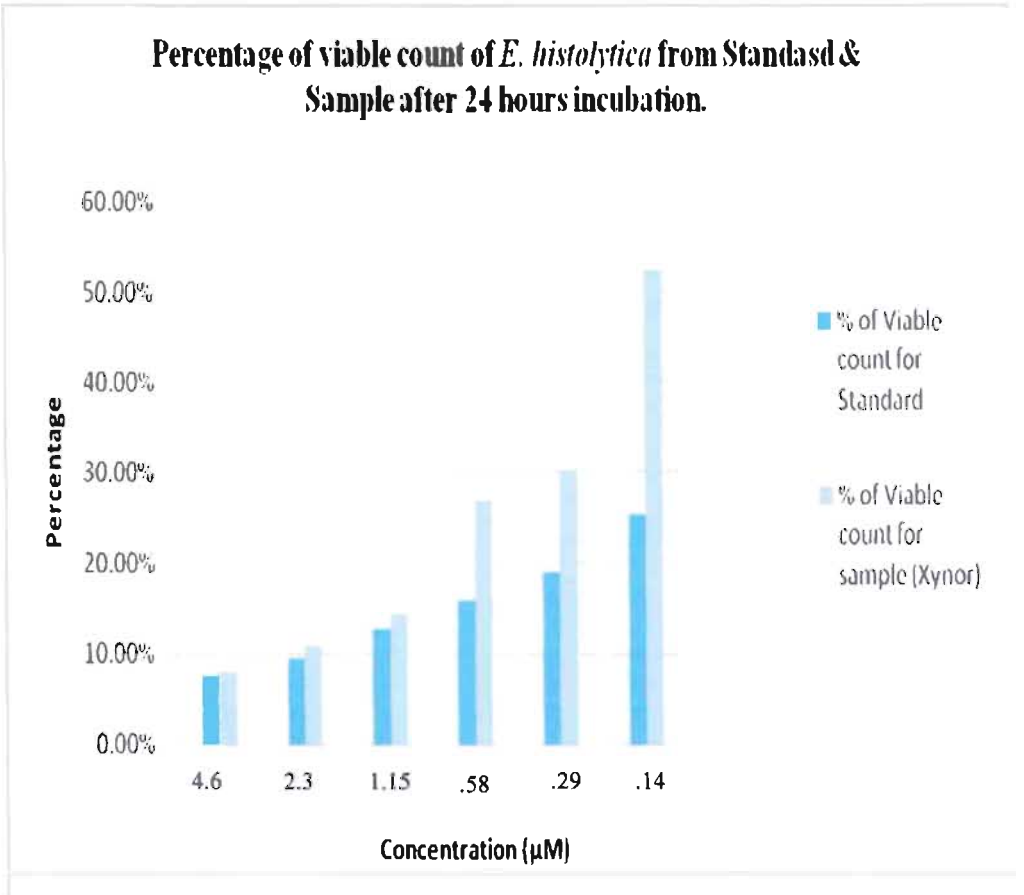

Figure-25: The concentration of viable count of *E. histolytica* from standard and brand (Xynor) after 24 hours incubation.

From the above graph it can be concluded that the percentage of viable count of *E. histolytica* for standard is 7.61% and for brand(Xynor) is 7.93 %.

3.3 Non viable count of clinically isolates *E. histolytica* from standard & commercial product of Ornidazole (Xynor) after 24 hours of incubation.

The initial count of the parasites were 1.575×10^5 mL^{-1}. After 24 hours ,the parasites count of the control were 3.95×10^5 mL^{-1}. The number of parasites were increased in the control after 24 hours of incubation.

Table-10: Non viable count *E. histolytica* from Standard & Brand (Xynor) after 24 hours of incubation.

Concentration of Ornidazole (μM) Standard	Non Viable count of *E. histolytica*	Concentration of Ornidazole (Xynor) (μM)	Non Viable count of *E. histolytica*
4.6	1.45×10^5 mL^{-1}	4.6	1.44×10^5 mL^{-1}
2.3	$1.42\times10^5 mL^{-1}$	2.3	$1.40\times10^5 mL^{-1}$
1.15	1.37×10^5 mL^{-1}	1.15	1.35×10^5 mL^{-1}
0.575	$1.32\times10^5 mL^{-1}$	0.575	1.15×10^5 mL^{-1}
0.288	$1.27\times10^5 mL^{-1}$	0.288	$1.10\times10^5 mL^{-1}$
0.144	$1.17\times10^5 mL^{-1}$	0.144	0.75×10^5 mL^{-1}

After 24 hours incubation ,when the concentration of Ornidazole was 0·144 μM the non viable count of *Entamoeba histolytica* were $1.17\times10^5 mL^{-1}$ for standard and 0.75×10^5 mL^{-1} for brand (Xynor) respectively. In the standard preparation , the non viable count of clinically isolates *Entamoeba histolytica* were $1.27\times10^5 mL^{-1}$, 1.32×10^5 mL^{-1}, $1.37\times10^5 mL^{-1}$ and $1.42\times10^5 mL^{-1}$ when the concentrations were 0.287, 0.575, 1.15 and 2.3 μM respectively. In case of Ornidazole brand (Xynor), the non viable count for clinically isolates *Entamoeba histolytica* were $1.10\times10^5 mL^{-1}$, 1.15×10^5 mL^{-1}, 1.35×10^5 mL^{-1} and $1.40\times10^5 mL^{-1}$ when the concentrations were 0.287, 0.575, 1.15 and 2.3 μM respectively. When the concentration of Ornidazole (standard) was increased to 4.6μM ,the non viable count of clinically isolates *Entamoeba histolytica* was increased to 1.45×10^5 mL^{-1} and for the Ornidazole brand (Xynor) ,the non viable count of clinically isolates *Entamoeba histolytica* was also increased to 1.44×10^5 mL^{-1}.

Table-11 : Non viable count of clinically isolates *E. histolytica* from Standard & Brand (Xynor) after 24 hours of incubation in percentage

Percentage of Ornidazole (µM) Standard	Non Viable count of *E. histolytica* in percentage	Percentage of Ornidazole(Xynor) (µM)	Non Viable count of *E. histolytica* in percentage
4.6	92.39 %	4.6	92.07 %
2.3	90.48 %	2.3	89.21 %
1.15	87.31 %	1.15	85.72 %
0.575	84.13 %	0.575	73.08 %
0.288	80.96 %	0.288	69.83 %
0.144	74.61 %	0.144	47.62 %

Figure 28 : Concentrations of non viable count of *E. histolytica* from standard & brand (Xynor) after 24 hours Incubation in percentage

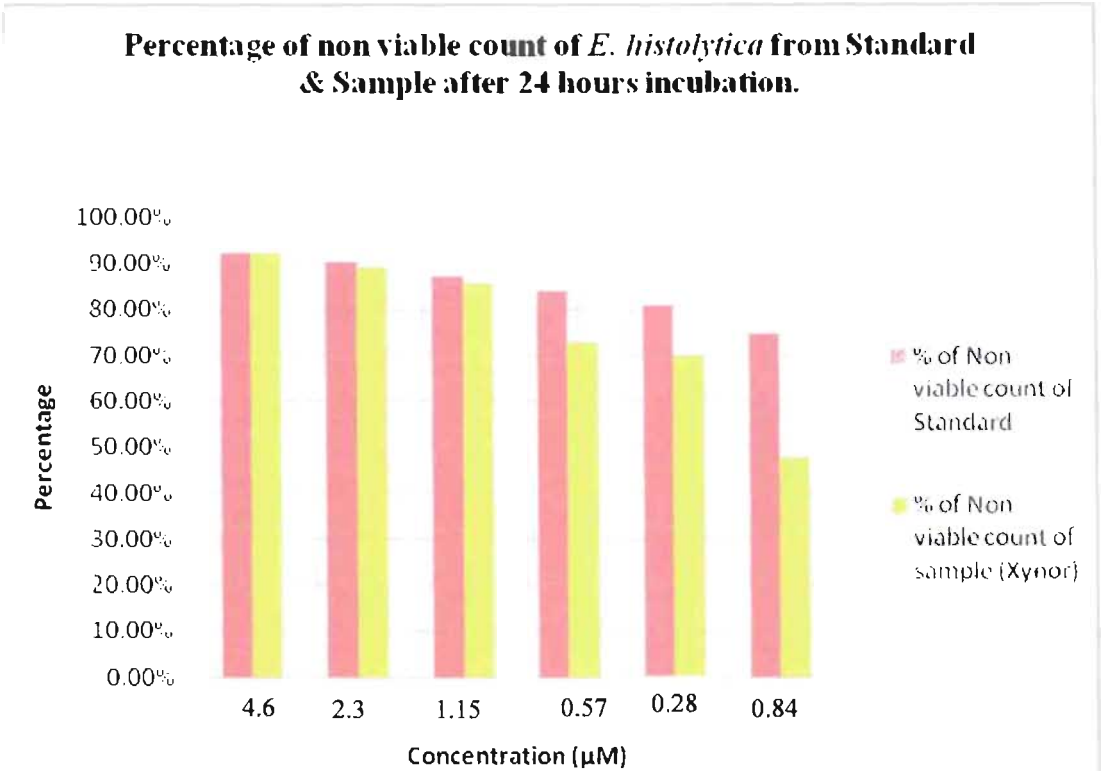

Figure 28: Concentrations of non viable count in percentage of *E. histolytica* from standard & brand (Xynor) after 24 hours incubation.

From the above graph it can be concluded that the percentage of non viable count of *E. histolytica* for standard is 92.39% and for brand(Xynor) is 92.07%.

3.4 Viable count of *E.histolytica* from standard & commercial product of Ornidazole (Troniz) after 24 hours incubation

The initial count of the parasites were 1.57×10^5 mL^{-1}. After 24 hours of incubation the parasites count of the control were 1.8×10^5 mL^{-1} . The number of parasites were increased in the control after 24 hours of incubation.

Table-12: Viable count of *E. histolytica* for Standard & commercial product of Ornidazole (Troniz) after 24 hours incubation.

Concentration of Ornidazole (μM) Standard	Viable count of *E. histolytica*	Concentration of Ornidazole (Troniz) (μM)	Viable count *E. histolytica*
4.6	0.22×10^5 mL^{-1}	4.6	0.25×10^5 mL^{-1}
2.3	0.47×10^5 mL^{-1}	2.3	0.45×10^5 mL^{-1}
1.15	0.57×10^5 mL^{-1}	1.15	0.6×10^5 mL^{-1}
0.575	0.67×10^5 mL^{-1}	0.575	0.7×10^5 mL^{-1}
0.288	0.95×10^5 mL^{-1}	0.288	1.0×10^5 mL^{-1}
0.144	1.0×10^5 mL^{-1}	0.144	1.2×10^5 mL^{-1}

After 24 hours incubation ,when the concentration of Ornidazole was 0·144 μM the viable count of *Entamoeba histolytica* were 1.0×10^5 mL^{-1} for standard and 1.2×10^5 mL^{-1} for brand (Troniz) respectively. In the standard preparation , the viable count of clinically isolates *Entamoeba histolytica* were 0.95×10^5 mL^{-1}, 0.67×10^5 mL^{-1} , 0.57×10^5 mL^{-1}, and 0.47×10^5 mL^{-1} when the concentrations were 0.287, 0.575, 1.15 and 2.3 μM respectively. In case of Ornidazole brand (Troniz) the viable count for clinically isolates *Entamoeba histolytica* were 1.0×10^5 mL^{-1}, 0.7×10^5 mL^{-1}, 0.6×10^5 mL^{-1} and 0.45×10^5 mL^{-1} when the concentration were 0.287, 0.575, 1.15 and 2.3 μM respectively. When the concentration of Ornidazole (standard) was increased to 4.6μM ,the viable count of clinically isolates *Entamoeba histolytica* was decreased to 0.22×10^5 mL^{-1} and for the Ornidazole brand (Troniz) ,the count of clinically isolates *Entamoeba histolytica* was decreased to 0.25×10^5 mL^{-1} .

Table-13: Percentage of viable count of *E. histolytica* from standard and commercial product (Troniz) of Ornidazole after 24 hours of incubation.

Concentration of Ornidazole (μM) Standard	Viable count of *E. histolytica* in percentage	Concentration of Ornidazole (Troniz) (μM)	Viable count of *E. histolytica* in percentage
4.6	14.01 %	4.6	15.92 %
2.3	29.29 %	2.3	28.66 %
1.15	36.30 %	1.15	40%
0.575	42.67 %	0.575	44.58%

0.288	60.50 %	0.288	63.69%
0.144	63.69 %	0.144	76.43 %

Figure 30 : Percentage of viable count of *E. histolytica* for standard and brand(Troniz) after 24 hours of Incubation.

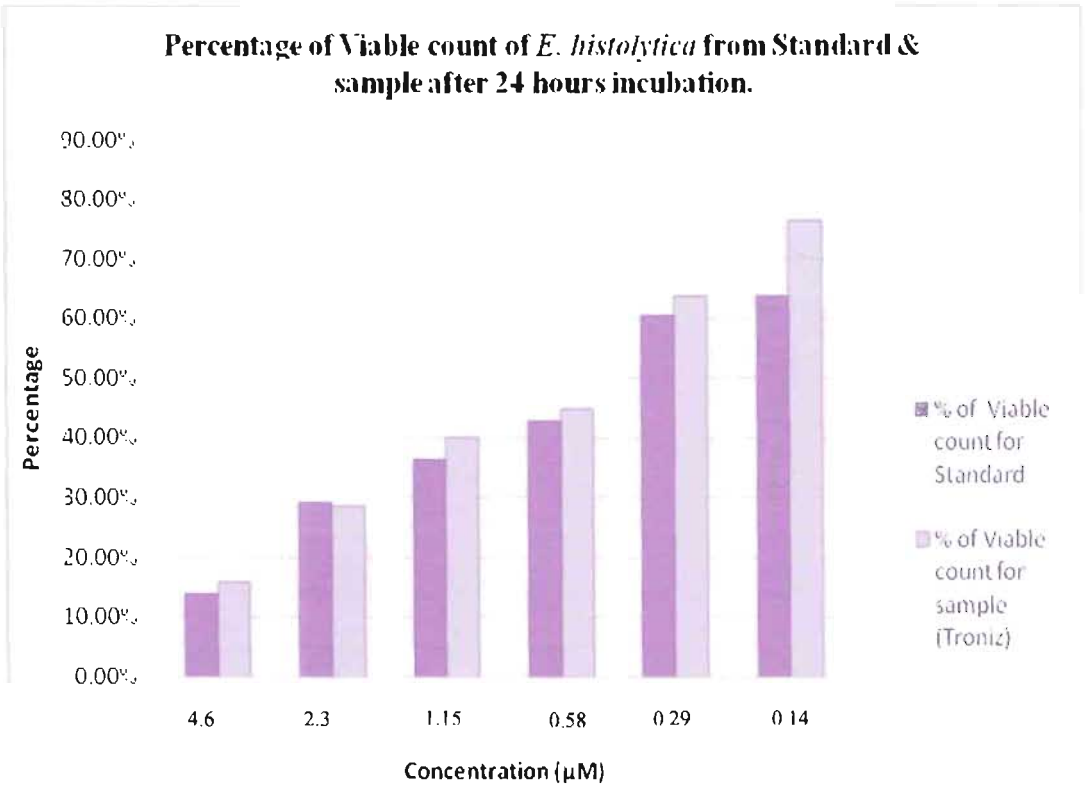

Figure 29: The percentage of viable count of *E. histolytica* for standard and brand(troniz) after 24 hours of incubation.

From the above graph it can be concluded that the percentage of viable count of *E. histolytica* for standard is 14.01 % and for brand(Xynor) is 15.92 %.

3.5 Non viable count of clinically isolates *E. histolytica* for standard and commercial product of Ornidazole (Troniz) after 24 hours of incubation

The initial count of the parasites were 1.57×10^5 mL^{-1}. After 24 hours the parasites count of the control were 1.8×10^5 mL^{-1} . The number of parasites were increased in the control after 24 hours of incubation.

Table-14 : Non viable count of *E. histolytica* from Standard and commercial product (Troniz) after 24 hours of incubation.

Concentration of Ornidazole (μM) Standard	Non Viable count of *E. histolytica*	Concentration of Ornidazole (Troniz) (μM)	Non Viable count of *E. histolytica*
4.6	1.35×10^5 mL^{-1}	4.6	1.2×10^5 mL^{-1}
2.3	1.10×10^5 mL^{-1}	2.3	1.12×10^5 mL^{-1}
1.15	1.0×10^5 mL^{-1}	1.15	$0{\cdot}97\times10^5 mL^{-1}$
0.58	0.9×10^5 mL^{-1}	0.58	0.87×10^5 mL^{-1}
0.29	0.62×10^5 mL^{-1}	0.29	0.57×10^5 mL^{-1}
0.14	0.57×10^5 mL^{-1}	0.14	$0.37\times10^5 mL^{-1}$

After 24 hours incubation ,when the concentration of Ornidazole was 0·144 μM the viable count of *Entamoeba histolytica* were 0.57×10^5 mL^{-1} for standard and $0.37\times10^5 mL^{-1}$ for brand (Troniz) respectively. In the standard preparation , the viable count of clinically isolates *Entamoeba histolytica* were 0.62×10^5 mL^{-1}, 0.9×10^5 mL^{-1} , 1.0×10^5 $mL^{-1}$and 1.10×10^5 mL^{-1} when the concentrations were 0.287, 0.575, 1.15 and 2.3 μM respectively. In case of Ornidazole brand (Troniz) the viable count for clinically isolates *Entamoeba histolytica* were 0.57×10^5 mL^{-1}, 0.87×10^5 mL^{-1}, $0{\cdot}97\times10^5 mL^{-1}$ and 1.12×10^5 $mL^{-1}$when the concentration were 0.287, 0.575, 1.15 and 2.3 μM respectively. When the concentration of Ornidazole (standard) was increased to 4.6μM ,the non viable count of clinically isolates *Entamoeba histolytica* was increased to 0.22×10^5 mL^{-1} and also for the Ornidazole brand (Troniz) , the non viable count of clinically isolates *Entamoeba histolytica* was increased to 1.2×10^5 mL^{-1} .

Table-15 : Percentage of non viable count of *E. histolytica* for standard & commercial product (Troniz) of Ornidazole after 24 hours incubation.

Concentration of Ornidazole (µM) Standard	Non Viable count of *E. histolytica in* percentage	Concentration of Ornidazole (µM) Drug(Troniz)	Non viable count of *E. histolytica in* percentage
4.6	85.98	4.6	80 %
2.3	70.06	2.3	74.66 %
1.15	63.69	1.15	61.78 %
0.575	57.32	0.575	55.4 %
0.288	39.49	0.288	36.30 %
0.144	36.30	0.144	23.56 %

Figure 30 : Percentage of non viable count of *E. histolytica* for standard and commercial product (Troniz) of Ornidazole after 24 hours incubation.

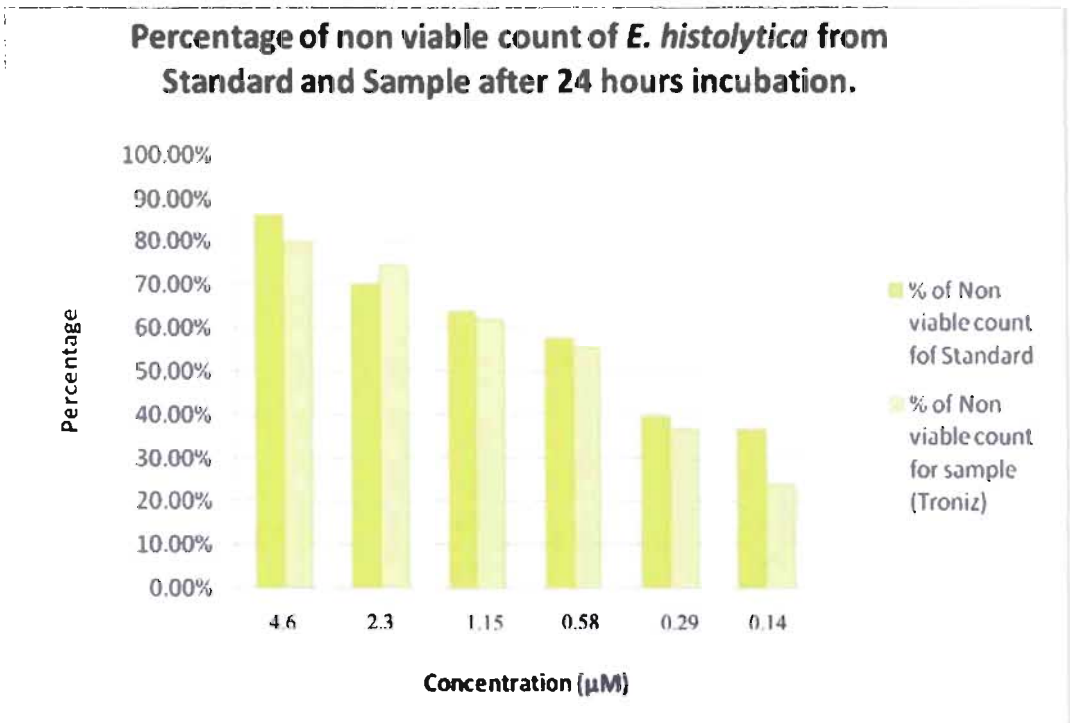

Figure 30: The percentage of non viable count of *E. histolytica* from standard and brand (Troniz) after 24 hours incubation.

From the above graph it can be concluded that the percentage of non viable count of *E. histolytica* for standard is 85.98 % and for brand(Troniz)

CHAPTER-4

DISCUSSION

4.1 Discussion

The objective of this study is to determine the therapeutic efficacy of Ornidazole and it's brand Troniz and Xynor , those are used against clinically isolates *Entamoeba histolytica* associated amoebiasis treatment.

Ornidazole, 5-nitroimidazole derivative is effective in the treatment of a broad range of parasitic infections. It is active against several protozoa, including *Cryptosporidium parvum*, *Blastocystis hominis*, *Entamoeba histolytica* and *Giardia intestinalis*. It is also used for the treatment of amoebiasis caused by *E. histolytica* . It is used for the treatment of infectious diarrhea caused by *Cryptosporidium parvum* and *Giardia lamblia* in patients 1 year of age and older.

Treatment failure among amoebiasis patients often raises the possibility of drug resistance (Ayala P, 1990). The incidence of amoebic dysentery is reduced to various synthetic drugs. It is surely observed that Ornidazole have high efficacy on inhibition of clinical isolates of *Entamoeba histolytica* which is the organism that is responsible for amebiasis.(Smaill F, Gastroenterol Can J ,2000)

The clinical isolates of *Entamoeba histolytica* were treated with different brand of Ornidazole at different concentrations. The experimental concentrations were 0.144, 0.29, 0.575, 1.15, 2.3 and 4.6 μM. A control group was made to measure the change in the viable counts and was put into the ELISA plate. Each ELISA plate now contained different concentration of Ornidazole and some amount of clinically isolates *Entamoeba histolytica* (100 micro liters). After that the preparation was incubated for 24 hours. Finally the viable and non viable parasites of *Entamoeba histolytica* were counted and recorded. It is found that the different brands of Ornidazole have good sensitivity against clinical isolates of *Entamoeba histolytica.*

Ornidazole shows 100% inhibition of *Entamoeba histolytica* when the concentration is more than 4.6 μM. In an experiment is named "*In vitro* studies on the sensitivity of local *Entamoeba histolytica* to anti-amoebic drugs" Ornidazole concentration is used 0.0625 to 0.25 microgram/ml, metronidazole ranged from 0.0625 to 0.125 microgram/ml, and tinidazole ranged from 0.0625 microgram/ml to 0.25 microgram/ml. The MIC of these

three drugs was significantly different. The MIC of Metronidazole against *E. histolytica* was superior than the other drugs. After that Ornidazole showed good result. But there was not so much different. (Chintana T. et al , 2001)

From the study it is observed that the percentage of non viable count of clinically isolates *E. histolytica* for standard is 92.39% and for brand (Xynor) is 92.07%. On the other hand the percentage of non viable count of clinically isolates *E. histolytica* for standard is 85.98 % and for brand(Troniz) is 80 %.

Here the two brands are complying with the results of the standard . But among the two brands Xynor is complying more with the result of standard (inhibition growth of *E. histolytica*) than the Troniz. That's why it can say that, Xynor is more effective for the treatment of amoebiasis than the troniz.

As the percentages of the non viable counts are increased when the concentrations of Ornidazole are increased. A control group is made to measure the change in the viable counts and in the non viable counts of *Entamoeba histolytica.* The lowest number of non viable count of *Entamoeba histolytica* is found in the control group and highest in the 4.6 μM concentration of Ornidazole.

The *in vitro* sensitivity of different brands of Ornidazole against *Entamoeba histolytica* is high and it is an innovative treatment option against amoebiasis. This study helps to make awareness of both physicians and consumers to select the right drug among the two brands .

CHAPTER-5

CONCLUSION

5.1 Conclusion

Amebiasis is a parasitic infection caused by the *Entamoeba histolytica.* Amebiasis is the third leading parasitic cause of morbidity and mortality due to parasitic disease in human after malaria and schistomiasis. On a global basis, amebiasis affects approximately 50 million persons each year, resulting in nearly 100,000 deaths. Ornidazole, 5-nitroimidazole derivative is an antiprotozoal agent. It is used for the treatment of amoebiasis.This study is carried to understand the effectiveness of different brands of Ornidazole against the *E. histolytica* . After the experiment it is observed that, Xynor (Beximco pharmaceytical) shows better effect than Troniz (Unimed &Unihealth pharmaceutical).

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