In vitro sensitivity study of Zoxanide against clinical isolates of *Entamoeba histolytica.*

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This paper is dedicated to my beloved parents

CERTIFICATE

This is to certify that the thesis "*In vitro* sensitivity study of Zoxanide 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 Zahidul Islam (ID: 2008-1-70-068) 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

Amebiasis is a very common disease all over the world. Almost 10% people of the world suffer from amoebiasis. Poverty, ignorance, overcrowding, poor sanitation and malnutrition favor transmission and increased disease burden. *Entamoeba histolytica* is the etiological agent of amoebic dysentery and amoebic liver abscess. The objective of this study was to find out the *in vitro* sensitivity of Nitazoxanide against clinical isolates of *Entamoeba histolytica*. *Entamoeba histolytica* isolates were collected from the stool and then cultured in different culture medium. Zoxanide was prepared in different concentrations of 4.6, 2.3 ,1.15 , 0.575 , 0.287 , 0.143 and 0.07187 μM using the Di-methyl sulphoxide (DMSO) solvent. They were incubated for 24 hours at 37°C. After incubation the isolates of viable *Entamoeba histolytica* were counted. The viable counts of the Entamoeba histolytica in each concentration of Zoxanide were compared to the initial concentration as well as the control. As the concentrations of Nitazoxanide decreases (4.6, 2.3, 1.15, 0.575, 0.287, 0.143 and 0.07187 μM), the inhibitory activity of Zoxanide was also found to be decreased. Further studies are needed to check the efficacy of other brands of Nitazoxanide.

Key words: Amebiasis, *Entamoeba histolytica*, Nitazoxanide, Viable, non viable.

CHAPTER 1:
INTRODUCTION

1.1 Introduction

Amebiasis is an infection of the intestines caused by the parasite *Entamoeba histolytica*. *It* can live in the large intestine (colon) without causing disease. However, sometimes, it invades the colon wall, causing colitis, acute dysentery, or long-term (chronic) diarrhea. The infection can spread through the blood to the liver and, rarely, to the lungs, brain, or other organs. This is generally contracted by water or any prepared foods infected due to the poor hygienic condition and that might cause contaminations. It is features by watery diarrhea, bloody stools and feverish conditions. This condition occurs worldwide, but it is most common in tropical areas with crowded living conditions and poor sanitation. Africa, Mexico, parts of South America, and India has significant health problems associated with this disease.

Entamoeba histolytica can also spread through food or water contaminated with stools. This contamination is common when human waste is used as fertilizer. It can also be spread from person to person- particularly by contact with the mouth or rectal area of an infected person.

Risk factors for severe amebiasis include:

- Alcoholism
- Cancer
- Malnutrition
- Older or younger age
- Pregnancy
- Recent travel to a tropical region
- Use of corticosteroid medication to suppress the immune system.

In many cases, the parasite lives in a person's large intestine without causing any symptoms. But sometimes, it invades the lining of the large intestine, causing bloody diarrhea, stomach pains, cramping, nausea, loss of appetite, or fever. In rare cases, it can spread into other organs such as the liver, lungs, and brain.

Amebiasis typically occurs in areas where living conditions are crowded and where there is a lack of adequate sanitation. The illness is very prevalent in parts of the

developing world, including Africa, Latin America, India, and Southeast Asia. It is rare in the United States, occurring mostly in immigrants, recent travelers to high-risk countries, and people with HIV/AIDS.

Although most cases of amebiasis are asymptomatic, dysentery and invasive extraintestinal disease can occur. Amebic liver abscess is the most common manifestation of invasive amebiasis, but other organs can also be involved, including pleuropulmonary, cardiac, cerebral, renal, genitourinary, and cutaneous sites. In developed countries, amebiasis primarily affects migrants from and travelers to endemic regions, men who have sex with men, and immunosuppressed or institutionalized individuals.

E histolytica is transmitted via ingestion of the cystic form (infective stage) of the protozoa. Viable in the environment for weeks to months, cysts can be found in fecally contaminated soil, fertilizer, or water or on the contaminated hands of food handlers. Fecal-oral transmission can also occur in the setting of anal sexual practices or direct rectal inoculation through colonic irrigation devices. Excystation then occurs in the terminal ileum or colon, resulting in trophozoites (invasive form). The trophozoites can penetrate and invade the colonic mucosal barrier, leading to tissue destruction, secretory bloody diarrhea, and colitis resembling inflammatory bowel disease. In addition, the trophozoites can spread hematogenously via the portal circulation to the liver or even to more distant organs.

Amebiasis is the third leading parasitic cause of death worldwide, surpassed only by malaria and schistosomiasis. On a global basis, amebiasis affects approximately 50 million persons each year, resulting in nearly 100,000 deaths.

Fedor Aleksandrovich Lošch, in St. Petersburg, Russia, first described amebiasis in 1875. He originally named the organism *Amoeba coli* and documented its pathogenicity in a dog fed with dysenteric stools from a patient. In 1886 in Egypt, Kartulis proved amebae to be the cause of intestinal and hepatic lesions in patients with diarrhea. In 1891, Councilman and Lafleur, at Johns Hopkins University Hospital, distinguished between bacillary and amebic dysentery. In 1913 in the Philippines, Walker and Sellards described the pathogenic role of amebae in extensive studies.

The parasite has 2 forms: a motile form, called the trophozoite, and a cyst form, responsible for the person-to-person transmission of infection. The trophozoite of *E histolytica* inhabits the large intestine to produce lesions of amebic colitis. Invasion of the colonic mucosa leads to dissemination of the organism to extracolonic sites, predominantly the liver. Faced with an adverse colonic environment, the trophozoite changes to the cystic form, better adapted to survival.

The trophozoite of *E histolytica* averages 25 mm, ranging from 10-60 mm. It has a clear ectoplasm and a somewhat granular endoplasm that contains several vacuoles. The trophozoite has a single 3-mm to 5-mm nucleus with fine peripheral chromatin and a central nucleolus. Ingested RBCs may be present within the trophozoite (Courtesy of Centers for Disease Control and Prevention).

The cyst of *E histolytica* averages 12 mm, ranging from 5-20 mm. It has 1-4 nuclei that are morphologically similar to the nuclei of the trophozoite. The cyst may have iodine-stainable glycogen clumps and chromatoid bodies with smooth rounded edges (Courtesy of Centers for Disease Control and Prevention.)

Most kids who get amebiasis have minimal or no symptoms. When children do become ill, they experience abdominal pain that begins gradually, along with frequent loose or watery bowel movements, cramps, nausea, and a loss of appetite. In some cases they develop a fever and, possibly, bloody stools.

For some people, symptoms of amebiasis can begin within days to weeks of swallowing food or water contaminated by amoebas. For other people, symptoms of amebiasis either take months to appear or never appear at all.

Some parasites are--

- *Dientamoeba fragilis,* which causes *Dientamoebiasis*
- *Entamoeba dispar*
- *Entamoeba hartmanni*
- *Entamoeba coli*
- *Entamoeba moshkovskii*
- *Endolimax nana*

1.2 Amebiasis

Amebiasis is a common disease caused by the protozoan *Entamoeba histolytica*. It is estimated that approximately 10% of the world's population is infected by the closely related parasites *E. histolytica*. 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).

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).

1.3 Sign and Symptoms

Most people with this infection do not have symptoms. If symptoms occur, they are seen 7 to 10 days after being exposed to the parasite. Usually, the illness lasts about 2 weeks, but it can come back if treatment is not given.

Mild symptoms:

- Abdominal cramps
- Diarrhea
    - ✓ Passage of 3 - 8 semiformed stools per day
    - ✓ Passage of soft stools with mucus and occasional blood
- Fatigue
- Excessive gas (Intestinal gas)
- Rectal pain while having a bowel movement (tenesmus)
- Unintentional weight loss

Severe symptoms:

- Abdominal tenderness
- Bloody stools
    - ✓ Passage of liquid stools with streaks of blood
    - ✓ Passage of 10 - 20 stools per day
- Fever
- Vomiting
- Stomach cramp
- Rectal pain
- Dysentery
- Colitis
- Liver abscess
- Lung abscess
- Loose stool
- Stomach pain
- Nausea
- Peritonitis
- Mucous diarrhea
- Abdominal discomfort (Professional Guide to Diseases, 2005).

1.4 Clinical Manifestation

- The most common clinical manifestation is intestinal amebiasis.
- Intestinal disease may be asymptomatic or have mild symptoms such as abdominal discomfort, flatulence, constipation, and occasionally diarrhea.
- Nondysenteric colitis is characterized by intermittent diarrhea and abdominal pain.
- Acute amebic colitis (dysenteric) is associated with grossly bloody stools with mucus, abdominal pain, and tenesmus (Fred H. et, al. 2011).

1. **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
- ✓ Colic and gaseous distension of the lower abdomen
- ✓ Nausea, flatulence, abdomnal distension and tenderness in the right iliac region over the colon (Fred H. et, al. 2011).

2. 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
- ✓ Mild toxemia, constant fatigue and lassitude
- ✓ Abdomen loses its elasticity when picked up between fingers
- ✓ On sigmoidoscopy, scattered ulceration with yellowish and erythematous border
- ✓ The gangrenous type (fatal cases) is characterized by the appearance of large sloughs of intestinal tissues in the stool accompanied by hemorrhage (Fred H. et, al. 2011).

3. Extraintestinal forms

- ✓ 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 (Fred H. et, al. 2011).

1.5 Laboratory Diagnosis of Amebiasis

The detection of *Entamoeba histolytica*, the causative agent of amebiasis, is an important goal of the clinical microbiology laboratory. To assess the scope of *E. histolytica* infection, it is necessary to utilize accurate diagnostic tools. As more is discovered about the molecular and cell biology of *E. histolytica*, there is great potential for further understanding the pathogenesis of amebiasis. Molecular biology-based diagnosis may become the technique of choice in the future because establishment of these protozoa in culture is still not a routine clinical laboratory process. In all cases, combination of serologic tests with detection of the parasite (by antigen detection or PCR) offers the best approach to diagnosis, while PCR techniques remain impractical in many developing country settings. The detection of amebic markers in serum in patients with amebic colitis and liver abscess appears promising but is still only a research tool. On the other hand, stool antigen detection tests offer a practical, sensitive, and specific way for the clinical laboratory to detect intestinal *E. histolytica*. All the current tests suffer from the fact that the antigens detected are denatured by fixation of the stool specimen, limiting testing to fresh or frozen samples (Polage, CR. et, al. 2011).

1.6 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 (Diamond L. S, 1978).

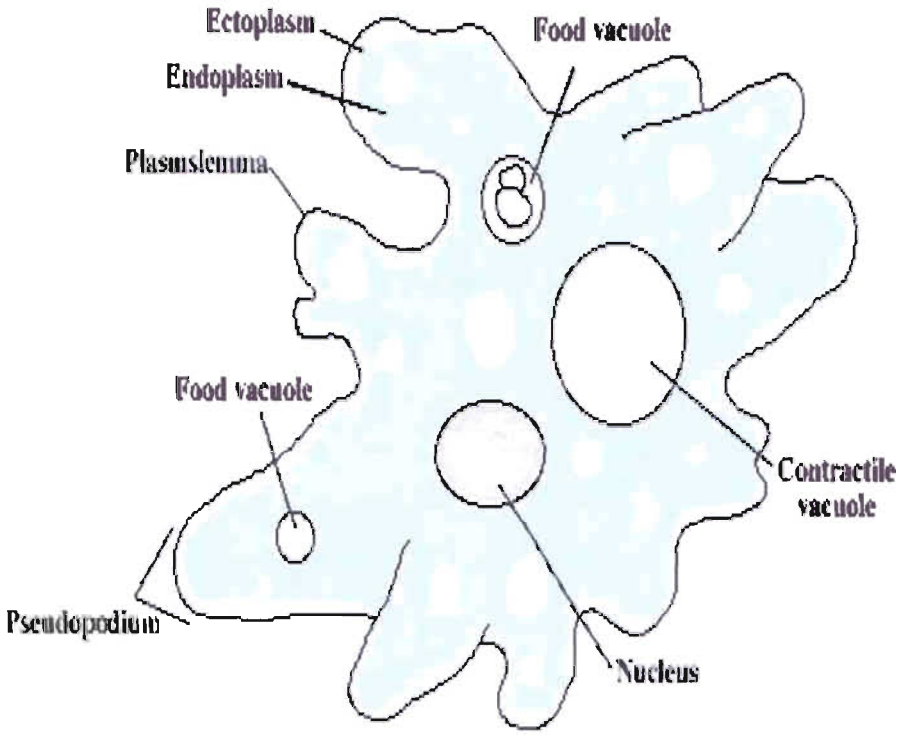

Figure 1: Amoeba proteus

Source: (www.google.com)

1.7 *E. histolytica*

Entamoeba histolytica is an anaerobic parasitic protozoan. If we classify it scientifically---

Common name: Ameba

Kingdom: Protista

Subkingdom: Protozoa

Phylum: Sarcomastigophora

Subphylum: Sarcodina

Class: Lobosea

Order: Amoebida

Family: Entamoebidae

Genus: *Entamoeba*

Species: Histolytica

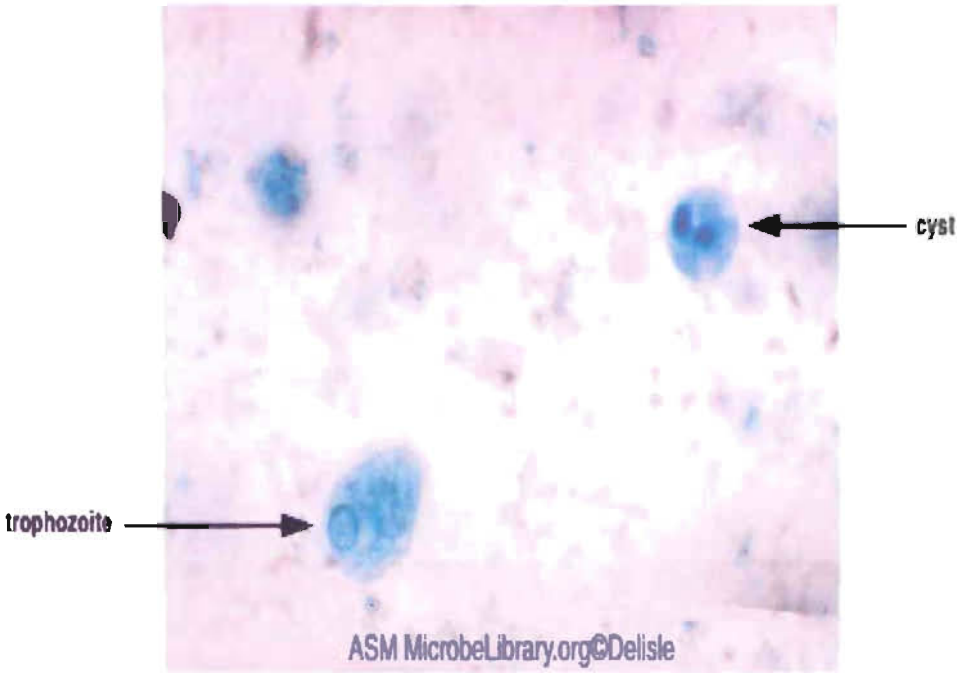

Figure 2: E histolytica

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. *Entamoeba histolytica* 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 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 trophozoiute to enter the host tissue. These can form large abcesses 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. If the intestinal tissue has been invaded the faeces can be bloody and diarrheic. Trophoziotes in diarrheic faeces are not stimulated to encyst because the faeces are not dehydrating. If they are not encysted they cannot long survive in the external environment. Secondary bacterial infection can complicate an already severe pathology. Accurate diagnosis of this parasite is important to prevent unnecessary treatment of a non-pathogenic strain, and to ensure treating a pathogenic strain. Definitive diagnosis is based on morphological characteristics of the trophozoites and cysts, the presence of erythrocytes in the trophozoites, and clinical sysmptoms (Ryan KJ et, al. 2004).

1.7.1 Structure

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 amebae can parasitize the human gastrointestinal tract and may cause diagnostic confusion. These include *Entamoeba hartmanni, Entamoeba gingivalis, Entamoeba coli, Endolimax nana*, and

Iodamoebabutschlii.

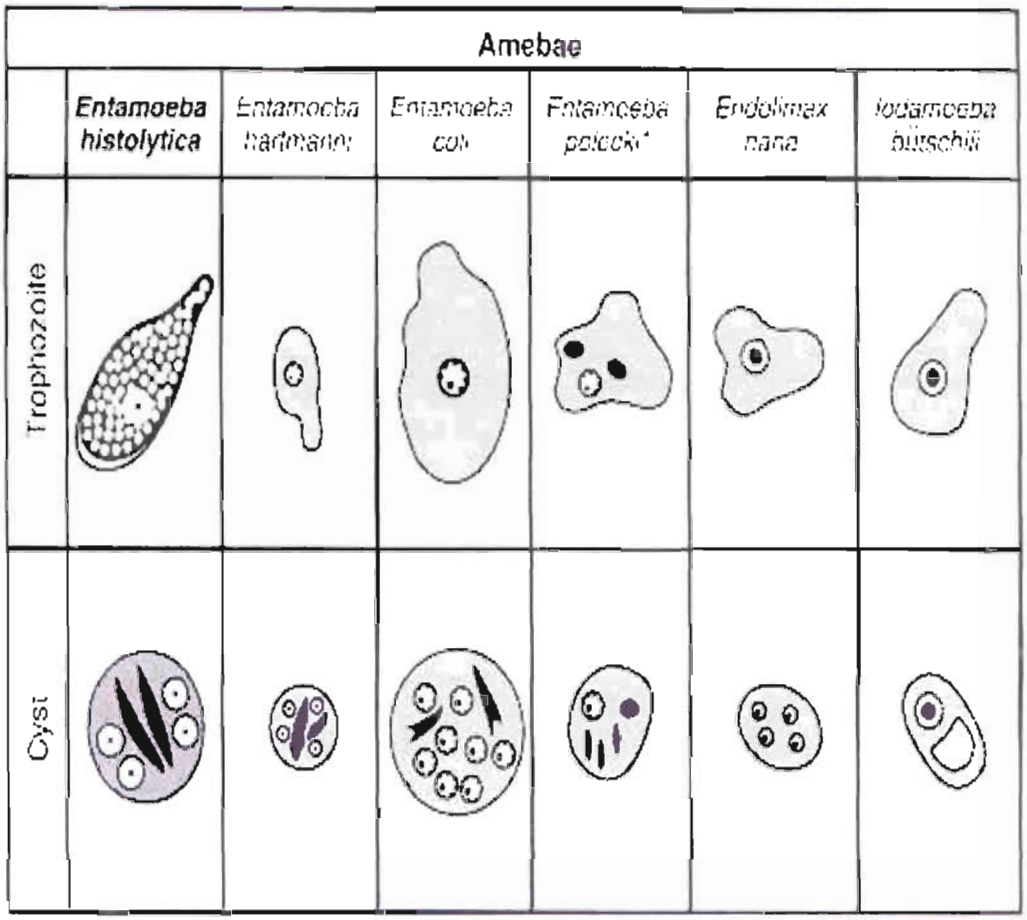

Figure 3: Amebas found in stool specimens of humans.

(Modified from Brooke, MM, Melvin DM: Morphology of diagnostic stages of intestinal parasites of man. Public Health Service Publication No. 1966, 1969.)

1.7.2 Classification

Many infections with *E histolytica* occur without evidence of invasion of the intestinal lining. Virulence in the amebathe ability to produce intestinal invasion or extraintestinal diseaseis a heritable characteristic. Morphologically identical amebas may be identified as pathogenic or non-pathogenic on the basis of size, cultural characteristics, virulence in a rat model or in tissue culture, selective agglutination by lectins, reaction

with monoclonal antibodies, or isoenzyme patterns. A pathogen-specific galactose adhesion epitope is described. Ribosomal RNA sequence analysis and restriction fragment length polymorphism analysis also can separate pathogenic from non-pathogenic strains.

A number of non-pathogenic but apparently genuine *E histolytic* strains have been isolated from human carriers. These amebas can be cultured at room temperature as well at 37° C and will grow in hypotonic media, whereas pathogenic amebas require isotonic media and 37° C for growth. These low-temperature strains have isoenzyme patterns identical with the sewage- associated, non-pathogenic *Entamoeba moshkovskii*. Two classic tests to identify pathogenic strains are the ability to cause cecal ulceration in weanling rats and agglutination by the lectin concanavalin A. These tests of virulence have been supplanted by isoenzyme analysis and the use of monoclonal antibodies to identify pathogenic strains of *E histolytica*, but the clinical applicability of this technique is pending.

Isoenzyme patterns are known for four amebic enzymes: glucose phosphate isomerase (GPI), hexokinase (HK), malate:NADP+ oxidoreductase (ME), and phosphoglucomutase (PGM). The isoenzyme patterns of three of these, GPI, HK, and PGM, can be used to define 20 zymodemes of *E histolytica*. The enzyme markers associated with pathogenicity are the presence of a b band and the absence of an a band for PGM.

Zymodemes II, VI, VII, XI, XII, XIII, XIV, XIX, and XX are pathogenic. Zymodemes II and XI are responsible for liver abscesses. There have been several reports of cultured amebas undergoing a change in zymodeme pattern after manipulation of associated bacterial flora. Attempts to reproduce these observations have not been successful. Zymodeme patterns are of epidemiologic and research interest but their limited availability makes them less useful clinically. A number of other factors, primarily environmental, that affect virulence are discussed below.

It is possible to distinguish with monoclonal antibodies the galactose-specific adhesions from pathogenic and non-pathogenic ameba. This offers the possibility of simplified laboratory determination of pathogenicity.

1.7.3 Characteristics of *Entamoeba histolytica*

Table 1.7.4.1: Characteristics of *Entamoeba histolytica*

Genus and Species	*Entamoeba histolytica*
Etiologic Agent of	Aoebiasis; 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
Pathogenesis	Lytic necrosis (it looks "flask-shaped" holes in Gastrointestinal tract sections (GIT)
Lab Diagnosis	Most common in Direct Fecal Smear (DFS) and staining (but does not allow identification to species level); Enzymeimmunoassay (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	
Pathognomonic/ 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

1.7.5 Life cycle of *E. histolytica*

Infection by Entamoeba histolytica occurs by ingestion of mature cysts in fecally contaminated food, water, or hands. Excystation occurs in the small intestine and trophozoites are released, which migrate to the large intestine. The trophozoites multiply by binary fission and produce cysts, which are passed in the feces. Because of the protection conferred by their walls, the cysts can survive days to weeks in the external environment and are responsible for transmission. (Trophozoites can also be passed in diarrheal stools, but are rapidly destroyed once outside the body, and if ingested would not survive exposure to the gastric environment.) In many cases, the trophozoites remain confined to the intestinal lumen (A: non-invasive infection) of individuals who are thus asymptomatic carriers and cysts passers. In some patients the trophozoites invade the intestinal mucosa (B: intestinal disease), or, through the bloodstream, extraintestinal sites such as the liver, brain, and lungs (C: extra-intestinal disease), with resultant pathologic manifestations. It has been established that the invasive and noninvasive forms represent separate species, respectively E. histolytica and E. dispar, which are morphologically indistinguishable.

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asymptomatic carriers and cysts passers. In some patients the trophozoites invade the intestinal mucosa (B: intestinal disease), or, through the bloodstream, extraintestinal sites such as the liver, brain, and lungs (C: extra-intestinal disease), with resultant pathologic manifestations. It has been established that the invasive and noninvasive forms represent separate.

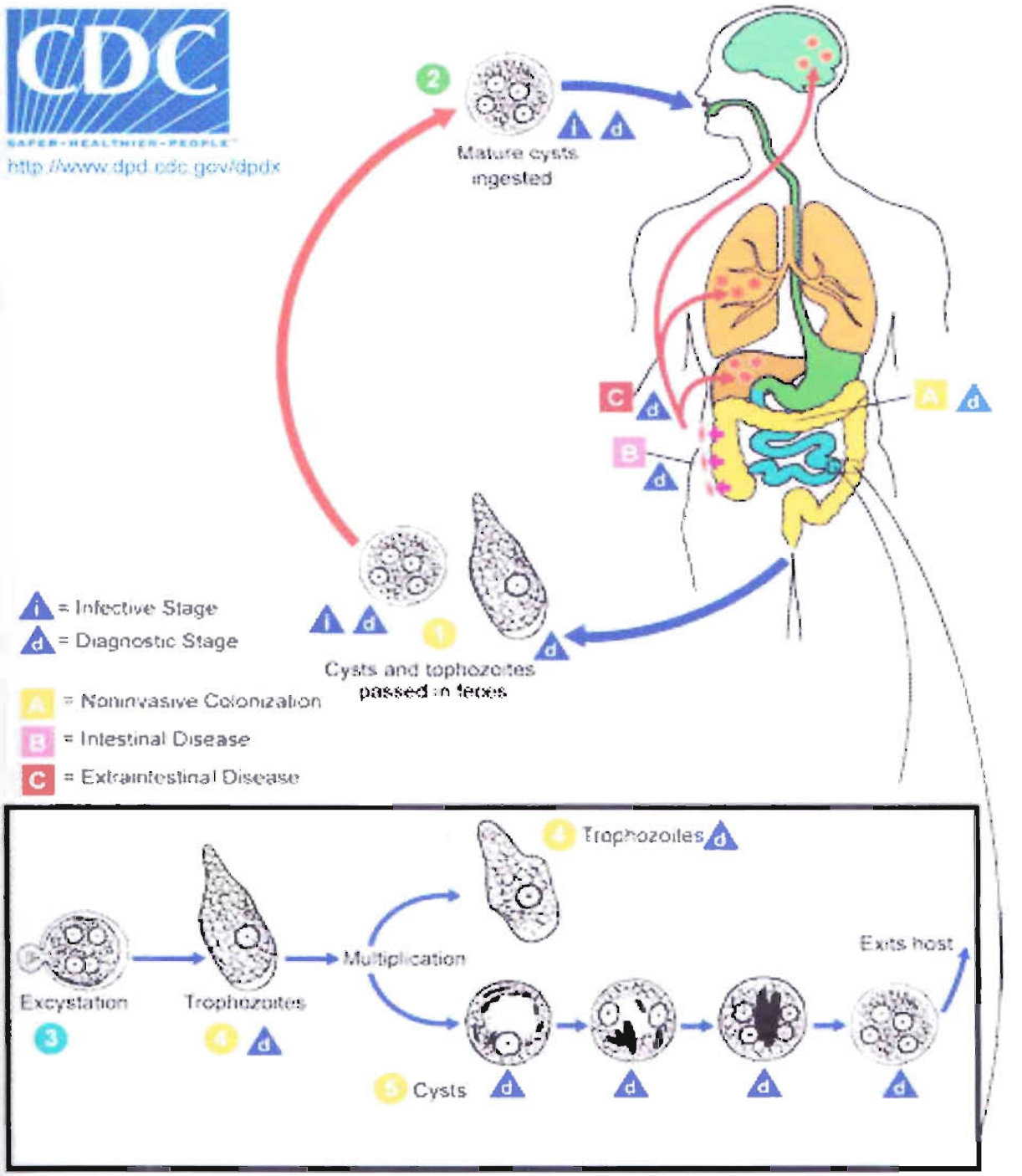


Figure 4: Life cycle of *Entameba histolytica*

If we see the flow chart below then it will be clearer

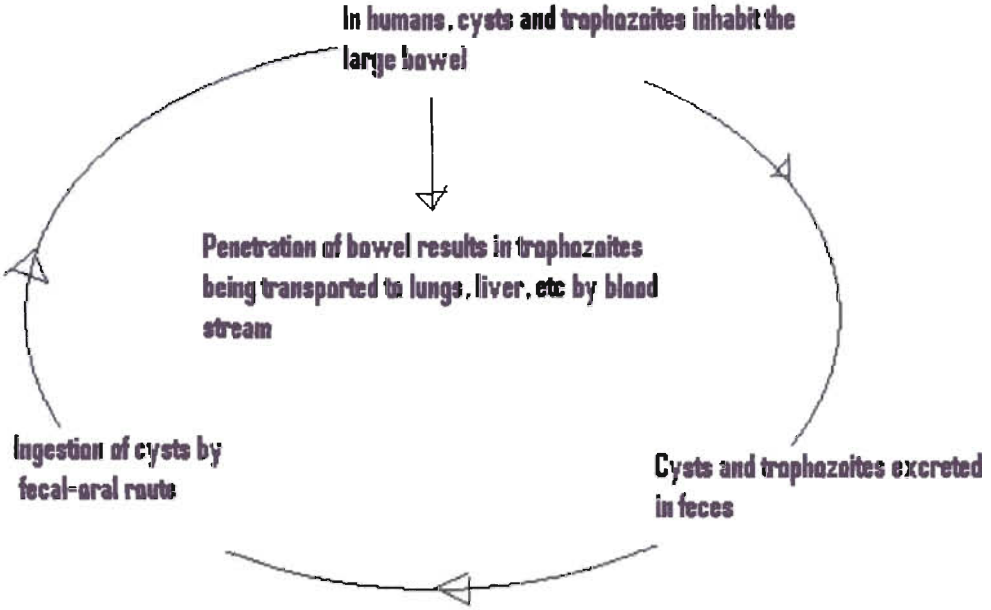


Figure 5: Life cycle of *Entameba histolytica*

1.8 Pathophysiology

E histolytica is a pseudopod-forming, nonflagellated protozoal parasite that causes proteolysis and tissue lysis (hence its name) and can induce host-cell apoptosis. Humans and perhaps nonhuman primates are the only natural hosts. Ingestion of *E histolytica* cysts from the environment is followed by excystation in the terminal ileum or colon to form highly motile trophozoites. Upon colonization of the colonic mucosa, the trophozoite may encyst and is then excreted in the feces or may invade the intestinal mucosal barrier and gain access to the blood stream and disseminate to the liver, lung, and other sites. Excreted cysts reach the environment to complete the cycle.

Disease may be caused by only a small number of cysts, but the processes of encystation and excystation are poorly understood. The adherence of trophozoites to colonic epithelial cells seems to be mediated by a galactose/*N* -acetylgalactosamine (GAL/GalNAc)–specific lectin. A mucosal immunoglobulin A (IgA) response against this lectin can result in fewer recurrent infections. Both lytic and apoptotic pathways have been described. Cytolysis can be undertaken by amoebapores, a family of peptides

capable of forming pores in lipid bilayers. Furthermore, in animal models of liver abscess, trophozoites induced apoptosis via a non-Fas and non–tumor necrosis factor-α1 receptor pathway. The amoebapores, at sublytic concentrations, can also induce apoptosis.

Cysteine proteinases have been directly implicated in invasion and inflammation of the gut and may amplify interleukin (IL)–1–mediated inflammation by mimicking the action of human IL-1–converting enzyme, cleaving IL-1 precursor to its active form. The cysteine proteinases can also cleave and inactivate the anaphylatoxins C3a and C5a, as well as IgA and immunoglobulin G (IgG).

Epithelial cells also produce various inflammatory mediators, including IL-1B, IL-8, and cyclooxygenase-2, leading to the attraction of neutrophils and macrophages. Corticosteroid therapy is known to worsen the clinical outcome, possibly because of its blunting effect on this innate immune response. Additional host defenses, including the complement system, could be inhibited directly by the trophozoites, suggested by the finding that a region of the GAL/GalNAc–specific lectin showed antigenic crossreactivity with CD59, a membrane inhibitor of the C5b-9 attack complex in human red blood cells. Trophozoites that reach the liver create unique abscesses with well-circumscribed regions of dead hepatocytes surrounded by few inflammatory cells and trophozoites and unaffected hepatocytes, suggesting that *E histolytica* are able to kill hepatocytes without direct contact. The genus *Entamoeba* contains many species, some of which (ie, *E histolytica, Entamoeba dispar, Entamoeba moshkovskii, Entamoeba polecki, Entamoeba coli, Entamoeba hartmanni*) can reside in the human interstitial lumen. *E histolytica* is, thus far, the only *Entamoeba* species definitely associated with disease; the others are considered nonpathogenic. More recent studies have recovered *E dispar* and *E moshkovskii* from patients with gastrointestinal symptoms, but a causal relationship is undetermined.

E dispar and *E histolytica* cannot be differentiated by direct examination, but recent molecular techniques established them as two different species, with *E dispar* being commensal (including in patients with HIV infection) and *E histolytica* pathogenic. In fact, it is now estimated that many individuals with *Entamoeba* infections are colonized with *E dispar*, which appears to be 10 times more common than *E histolytica*. However, in certain regions (eg, Brazil, Egypt), asymptomatic *E dispar* and *E histolytica*

infections are equally prevalent. In Western countries, approximately 20%-30% of men who have sex with men are colonized with *E dispar*.

1.9 Pathogenesis

The fecal-oral transmission of the ameba usually involves contaminated food or water. The parasite can also be transmitted directly by ano-genital or oro-anal sexual contact. Latent infections can become invasive in a setting of impaired host immunity.

Ingested cysts of *E histolytica* excyst in the small intestine. Trophozoites are carried to the colon, where they mature and reproduce. The parasite may lead a commensal existence on the mucosal surface and in the crypts of the colon. Successful colonization depends on factors such as inoculum size, intestinal motility, transit time, the presence or absence of specific intestinal flora, the host's diet and the ability of the ameba to adhere to the colonic mucosal cells. The ameba adherence molecule has been identified as a lectin which can bind to either of two common carbohydrate components of cell membrane, galactose and *N*-acetyl glactoseamine. Binding to colonic mucins blocks adherence to mucosal cells. Depletion of mucus results in binding to the mucosa, an essential step in the development of the disease. If amebas pass down the colon they encyst under the stimulus of desiccation, and then are evacuated with the stool.

The processes interactions which are thought to influence, or are implicated in, pathogenesis are described below:

I. 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. Axenic *E. histolytica* which have lost virulence can regain it if associated with bacteria like *Escherichia coli, Salmonella typhosa* or *S. paratyphi.* Bacterial strains which do not attach to, and get ingested by, trophozoites do not affect virulence. Virulence of trophozoites of strain 200: NIH varied depending on culture associates. When cultured with NRS bacteria or rabbit intestinal flora, these trophozoites caused acute disease in animals but very little disease when cultured with *Trypanosoma cruzi.* Reassociation with rabbit flora returned their infectivity. Wittner and Rosenbaum (1970) showed that direct association of *E. histolytica* with viable bacteria was required for virulence. Heat killed or

glutaraldehyde-fixed bacteria do not increase virulence. Soluble bacterial factors were not implicated. Bracha and Mirelman (1984) showed that *E. histolytica* exposed to live bacteria (that are known to adhere amoeba) for 30 min, increased in virulence in *In vivo* measurement, however it appears that association with bacteria is not an absolute requirement for invasion by *E. histolytica.* Association of specific bacteria with *E. histolytica* could change the architecture of the cell surface leading to altered properties of the cell (Bhattacharya *et al* 1992).

ii. Adherence to establish direct contact between trophozoite and target cell :

Adherence of trophozoites to target cells is a necessary prerequisite for cytotoxicity. Evidence for this is provided by the following observations. Cinemicrography of amoeba interacting with CHO cells on a glass cover slip showed that the CHO cells in direct contact with amoeba displayed membrane bleebbing and release from cover slip, while those not in direct contact, remained viable. When CHO cells and trophozoites were mixed and incubated in the presence of high molecular weight dextran (10%), lysis did not occur as dextran prevented adherence of trophozoites to target cells. In another experiment erythrocytes and trophozoites were mixed so as to allow adherence. Cells were centrifuged through a Ficoll gradient. Trophozoites that banded on top of the gradient had not adhered to erythrocytes. These were found to be much less virulent in a hamster liver model. Adherence to CHO cells at 37°C is inhibited by cytochalasins B and D, implicating the need for intact amoebic microfilament function in the process (Ravdin and Guerrant 1981).

Adherence is also inhibited by the Ca2+ channel blocker, Bepridil possibly by preventing intracellular Ca2+ flux which is thought to be necessary for microfilament function. Two surface molecules responsible for adherence have been identified one inhabitable by galactose or N-acetyl-D-galactosamine (GalNAc) and the other inhibitable by N-acetyl-D-glucosamine (GlcNAc) polymers (Petri, et, al. 1987). Pretreatment of amoeba with galactose or GalNAc inhibits adherence whereas pretreatment with neuraminic acid, maltose, mannose and GlcNAc has no effect. The Gal/GalNAc inhabitable lectin of *E. histolytica* has been characterized in considerable detail. The following data suggest that this molecule plays an essential role in amoebic adherence to target cells

(i) binding of trophozoites to CHO cells was inhibited 90-95% by 50 mM galactose and GalNAc while other sugars had no effect (Ravdin, 1981),

(ii) a mutant of CHO cell defective in production of N and O linked galactose-terminal oligosaccharides was almost completely resistant to adherence,

(iii) complex branched polysaccharides containing galactose groups at their termini were 1,000-fold more effective by weight than galactose, in inhibiting adherence to CHO cells (Petri *et al* 1987). The lectin has a molecular weight of 260 kDa and dissociates into heavy (170 kDa) and light (35-31 kDa) subunits in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Petri, et, al. 1989).

Three genes (*hgl* 1-3) encoding the 170 kDa subunit have been identified and characterized. Analysis of reduced amino acid sequences of the three genes indicates that this subunit of the lectin is a transmembrane protein. Northern lot analyses show that all the three genes are expressed in *E. histolytica* and the mRNAs were of the same size (4·0 kb).

Two light subunit genes (*hgl* 1-2) have also been identified and characterized. These genes have hydrophobic amino- and carboxy-terminal signal sequences. The 31 kDa isoform of the light subunit has a putative glycosylphosphatidylinositol (GPI) anchor cleavage/addition site while the 35 kDa isoform seems to lack it. Lectin heterodimers have been identified by two dimensional gel electrophoresis. The purified lectin showed atleast two major heterodimers, one containing the 170 kDa subunit.with 35 kDa isoform and another 170 and 31 kDa isoform. Minor heterodimers with 160 and 150 kDa heavy subunit isoforms were also present. The native lectin probably exists as oligomers of 400 kDa and 660 kDa. Apart from its function in adherence the lectin appears to mediate amoebic resistance to complement lysis.

iii. 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. Cytolysis of the target cell is thought to require amoebic microfilament function, Ca2+ flux and phospholipase A, among others. Microfilament

function seems to be necessary because lysis is inhibited at 25°C, a temperature at which actin gelation ceases; the optimal temperature being 37°C. (Pollard 1976)

Studies with the Ca2+-binding fluorescent dye FURA-2 showed 20-fold increase in intracellular Ca2+ in target cells within seconds of direct contact. Actual cell death occurred 5-15 min after the lethal hit. Possible roles of Ca2+ are in contact dependent release of cytotoxic enzymes and toxins, cytoskeletal changes and activation of Ca2 +-dependent enzymes, for example, phospholipases. Bos (1979) proposed that *E. histolytica* has two ways of killing host cells one is a rapid process occurring at close contact; other is slow, operating through soluble substances. Contact dependent cytolethal effect of *E. histolytica* is not inhibited by serum but contact-independent effect is inhibited. Lushbaugh et al. (1978a, b) showed that cell-free extracts from axenically grown trophozoites caused cytopathic effect on cell cultures, in the absence of serum. Lushbaugh *et al* (1979) and Bos (1979) independently purified a "cytotoxic" substance from trophozoite extracts which caused cell rounding and release from monolayer. The activity was associated with a protein (34-40 kDa) activated by thiols. It is believed that these thiol-proteases may be one of the molecules involved in pathogenesis. This is based on the fact that there seems to be a correlation between clinical severities with the level of thiol protease in clinical isolates (Reed *et al* 1989).

HM- 1: IMSS (more virulent of the two strains) has greater thiol protease activity than HK-9 strain. Patients with invasive disease produce antibodies against this enzyme; those with non-invasive disease do not. The enzyme has broad substrate specificity. It can utilize casein, gelatin, insulin, type I collagen, fibronectin and laminin as substrates.

It is a cathepsin B-like enzyme. Similar enzymes are found in extracellular milieu of invasive tumour cells. The protease may assist trophozoite to gain access to target cells by degrading the extracellular matrix.

A candidate for the toxin responsible for cytolysis may be a pore-forming peptide. Various amoebic pore-forming proteins (30, 14 and 5 kDa proteins) have been described (Dodson and Petri 1994). A 30 kDa amoebic protein was purified and shown to lyse erythrocytes and insert into and create pores in lipid bilayers. A 14 kDa poreforming protein was described as an ion-channel forming protein. Of these the 5 kDa protein (amoebapore) has been the best characterized. The primary structure of the 5 kDa amoebapore from pathogenic *E. histolytica* was determined by sequencing the purified peptide and the corresponding cDNA. It is composed of 77 amino acids, including 6

cysteine residues. Like other membrane-penetrating polypeptides, it too has an all α helical conformation. The cellular immune response of the host may contribute to destruction of the local host tissue. In hamster liver model recruitment of neutrophils is the initial host response to *E. histolytica* infection. Neutrophils are lysed when they come in contact with *E. histolytica* trophozoites releasing toxic products which lyse distant hepatocytes (Salata and Ravdin 1986).Leukocytes have the potential to lyse *E. histolytica* trophozoites and *vice versa* .*E. histolytica* is cytolytic to human leukocytes on contact. Only virulent amoeba can analyse polymorphonuclear leukocytes (PMNs) and lysis is blocked by GalNAc. At a ratio of 1000 PMNs per amoeba, trophozoites of the highly virulent strain HM-1:IMSS were not killed but those of the less virulent strain 303 were killed. At a ratio of 100 PMNs per amoeba, HM- 1: IMSS trophozoites killed a high percentage of PMNs while killing was less with 303 trophozoites. *E. histolytica* could kill macrophages and T lymphocytes *In vitro.* Conversely, macrophages activated with concanavalin A could kill amoeba. T lymphocytes from immune individuals, following incubation with amoebic antigen, were capable of killing *E. histolytica* trophozoites (Salata and Ravdin, 1985).

iv. Phagocytosis:

Trophozoites from stools of many invasive patients contain ingested erythrocytes and have much higher rate of erythrophagocytosis than healthy human carrier. Phagocytosis of mammalian tissue culture grown cells was observed by transmission electron microscopy. Cells with intact plasma membrane were phagocytosed, showing that prior cell lysis was not required for endocytosis. A phagocytosis deficient mutant of *E. histolytica* has been isolated by Orozco *et al* (1983). This mutant apart from being poor in phagocytosis, was also found to be low in virulence, when tested in the hamster liver model. Thus there seems to be a correlation between phagocytosis and virulence.

1.10 Epidemiology

Amebiasis is responsible for approximately 100,000 deaths per year, mainly in Central and South America, Africa, and India, as well as for considerable morbidity manifested as invasive intestinal or extraintestinal clinical features. Worldwide, amebiasis is the third most common cause of death due to parasitic infection after malaria and schistosomiasis, as estimated by the World Health Organization. Amebiasis infections are endemic in most temperate and tropical climates in the developing world. In some tropical countries, antibody prevalence rates (reflecting past or recent infection) exceed 50%. The

prevalence of amebiasis varies with the population of individuals affected, differing between countries and between areas with different socioeconomic conditions. Sometimes up to 50% of the population is affected in regions with poor sanitary conditions. It is thought that amebiasis directly affects over 50 million people, causing loss of manpower and subsequent economic damage.

In industrialized countries, amebiasis occurs in sexually active homosexual men, immigrants, tourists who travel to areas of endemic infection, institutionalized persons, and human immunodeficiency virus HIV-positive individuals. The overall prevalence of *E. histolytica* infection in industrialized countries such as the United States has been estimated to be 4% per year in spite of the presence of some high-risk groups. *E. histolytica* and *E. dispar* have traditionally been classified by isoenzyme analysis. Nowadays, in addition to this technique, typing by using monoclonal antibodies to surface antigens (antigen-based enzyme-linked immunosorbent assay [ELISA]), PCR-specific analysis, and restriction fragment length polymorphism (ribotyping) have been of great value in understanding the epidemiology of these parasites and in investigating disease outbreaks. Epidemiological studies have shown that low socioeconomic status and unsanitary conditions are significant independent risk factors for infection. In addition, people living in developing countries have a higher risk and earlier age of infection than do those in developed regions. For example, in Mexico, 11% of the tested population aged 5 to 9 years was infected, with the prevalence of infection being higher in girls (9.34%) (Clark, C. G et al. 2000).

1.11 Contagiousness

Amebiasis is contagious. Wherever living conditions are unsanitary and hygiene is poor, the chances are higher that the infection will pass from person to person.

Someone carrying amoebas in his or her intestines can pass the infection to others through the stool. When infected stool contaminates food or water supplies, amoebiasis can spread quickly too many people at once. This is especially true in developing countries where drinking water may be contaminated.

Amebiasis can also be spread between people through inadequate hand washing, by using the same objects, and by sexual contact.

1.12 Host defense

While infection is associated with suppression of cell-mediated, immunity, drug-cured patients are resistant to reinvasion by amoebae. Macrophages are the principal effector cells in host defence against E. histolytica via production of nitric oxide which is cytotoxic for the parasite. The objective of this study was to determine the T cell cytokine responses associated with host defence against E. histolytica . A mixed Th1/Th2 (Th0) response predominated at days 5--10 of amoebic liver abscess development in gerbils, as indicated by spleen and hepatic lymph node cell IL-2 (Th1 marker) and IL-4 (Th2 marker) production. However, T cell responses were profoundly suppressed at day 20 of infection. Serum collected at day 20, but not at other times, markedly suppressed T cell proliferative responses by inhibiting IL-2 production. A switch to a Th1 response occurred after day 20 of infection. Following drug-abbreviation of infection at day 20, animals were completely resistant to challenge infection in the liver and demonstrated a Th1 response. The Gal-lectin 170-kDa heavy subunit of E. histolytica is a protective antigen in gerbils and a potential subunit vaccine candidate. We determined which region of the Gal-lectin stimulates IL-12 production, as IL-12 is key to inducing Th1 cytokine responses. Native Gal-lectin plus interferon-gamma stimulated IL-12 p40 and p35 gene transcription and IL-12 p70 protein production in human macrophages. Using a panel of anti-170-kDa subunit monoclonal antibodies in inhibition studies, aa 596--998 was identified as the IL-12-inducing domain. These results suggest that this portion of the Gal-lectin has potential for use as a subunit vaccine to induce Th1-mediated immunity against E. histolytica (Huston, C. D et al. 2000).

1.13.1 Luminal host-defense mechanisms against invasive amebiasis:

Most humans infected with the virulent protozoan parasite *Entamoeba histolytica* do not develop invasive disease. Available evidence indicates that beneficial bacteria and the mucus gel layer in the colon lumen protect the host mucosa. Glycosidases produced by some normal colonic bacteria and luminal proteases degrade the key adherence lectin on *E. histolytica* trophozoites and decrease their adherence to epithelial cells. The mucus gel layer prevents those trophozoites that escape the hydrolases from reaching the epithelial cells. Trophozoite mucosal invasion is triggered only when both protective mechanisms are lost, as might occur during an unrelated pathogenic enteric bacterial

infection.

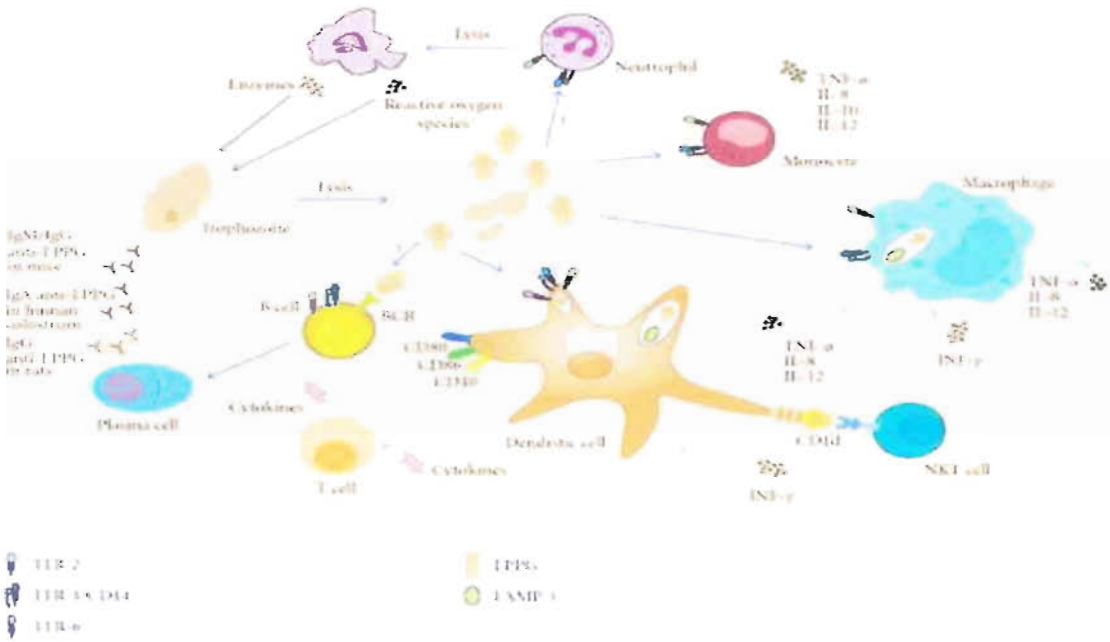


Figure 6: Host defense against E histolytica.

Source: Mann B.J.; 200

1.12.2 *Entamoeba histolytica* induced dephosphorylation in host cells:

Activation of host cell protein tyrosine phosphatases (PTPases) and protein dephosphorylation is an important mechanism used by various microorganisms to deactivate or kill host defense cells. To determine whether protein tyrosine dephosphorylation played a role in signaling pathways affecting *Entamoeba histolytica* mediated host cell killing , we investigated the involvement of PTPases during the attachment of *E. histolytica* to target cells. We observed a rapid decrease in cellular protein tyrosine levels in Jurkat cells, as measured with an antiphosphotyrosine monoclonal antibody, following adherence to *E. histolytica*. Ameba-induced protein dephosphorylation was contact dependent and required intact parasite, since blocking amebic adherence with galactose inhibited tyrosine dephosphorylation and amebic lysates had no effect on phosphotyrosine levels. Moreover, disruption of amebic adherence with galactose promoted recovery of phosphorylation in Jurkat cells, indicating that dephosphorylation precedes target cell death. The evidence suggests that ameba induced

dephosphorylation is mediated by host cell phosphatases. Prior treatment of Jurkat cells with phenylarsine oxide, a PTPase inhibitor, inhibited ameba-induced dephosphorylation. We also found proteolytic cleavage of the PTPase 1B (PTP1B) in Jurkat cells after contact with amebae. The calcium-dependent protease calpain is responsible for PTP1B cleavage and enzymatic activation. Pretreatment of Jurkat cells with calpeptin, a calpain inhibitor, blocked PTP1B cleavage and inhibited ameba-induced dephosphorylation. In addition, inhibition of Jurkat cell PTPases with phenylarsine oxide blocked Jurkat cell apoptosis induced by E. histolytica. These results suggest that *E. histolytica*-mediated host cell death occurs by a mechanism that involves PTPase activation.

1.13 Methods of Prevention:

1. Health education
2. Sanitary disposal of feces
3. Protect, chlorinate, and purify drinking water
4. Observe scrupulous cleanliness in food preparation and food handling
5. Detection and treatment of carriers
6. Fly control (they can serve as vector)

1.13.1 Prevention

To help prevent the spread of amoebiasis around the home:

- Wash hands thoroughly with soap and hot running water for at least 10 seconds after using the toilet or changing a baby's diaper, and before handling food.
- Clean bathrooms and toilets often; pay particular attention to toilet seats and taps.
- Avoid sharing towels or face washers.

To help prevent infection:

- Avoid raw vegetables when in endemic areas, as they may have been fertilized using human feces.
- Boil water or treat with iodine tablets.

- Avoid eating Street Foods especially in public places where others are sharing sauces in one container.

Good sanitary practice, as well as responsible sewage disposal or treatment, are necessary for the prevention of *E.histolytica* infection on an endemic level. *E.histolytica* cysts are usually resistant to chlorination; therefore sedimentation and filtration of water supplies are necessary to reduce the incidence of infection

1.17 Treatment and Control

E. histolytica is anaerobic and is sensitive to metronidazole when in the trophozoite form. Metronidazole is not effective against the cyst form of the organism, and therefore is followed up with iodoquinol or paromomycin to target the cysts. Dehydroemetine, a treatment that requires hospitalization due to the need for close supervision, and Diloxanide furoate, which is used in conjunction with other treatments in systematic cases, are only available through the Center for Disease Control and Prevention. If liver infection occurs Cholorquine may be used, in the event that Metronidazole is ineffective.

Entamoeba histolytica is a typical example of diseases that impact poor populations in developing countries. This makes it far more difficult to fund research and development for new treatments or vaccines, in spite of the fact that research has shown some possibilities for vaccines. The presence of IgA antibodies against E. histolytica indicates that a vaccine that brings about a mucosal immune response could be effective. However, the lack of projected profit limits the interest of pharmaceutical and biotechnology companies. Sanitation and hygiene are effective controls but often cannot be applied in many poor nations. Until a vaccine is created and distributed, Entamoeba histolytica will remain an important disease in mortality rates, especially among children in developing countries (Graf J, 2010).

1.14.1 Medical Care

Most individuals with amebiasis may be treated on an outpatient basis. Several clinical scenarios may favor inpatient care, as follows:

- Severe colitis and hypovolemia requiring intravenous volume replacement

- Liver abscess of uncertain etiology or not responding to empirical therapy
- Peritonitis and suspected amebic liver abscess rupture (Stanley SL, 2003).

1.14.2 Surgical Care

- Prompt surgical evaluation is needed in suspected cases of peritonitis, or perforated viscus.
- Surgical intervention is usually indicated in different clinical scenarios. In uncertain diagnosis the main concern is bacterial suprainfection in amebic liver abscess. If it is fail then respond to metronidazole after 4-day treatment duration. In empyema after amebic liver abscess rupture then the large left-sided amebic liver abscess representing the risk of rupture in the pericardium and severely ill patient with imminent amebic liver abscess rupture.
- Surgical drainage of uncomplicated amebic liver abscess is generally unnecessary and should be avoided.
- Percutaneous catheter drainage should be used judiciously in the setting of localized intra abdominal fluid collections. Although controversial, it might be used to aspirate large amebic liver abscesses (Stanley SL, 2003).

1.14.3 Consultations

- Infectious disease specialist
- General surgeon
- Gastrointestinal specialist.

1.14.4 Medication

- Asymptomatic amebiasis should be treated with a luminal agent (iodoquinol, paromomycin, diloxanide furoate) to eradicate infection. This recommendation is based on two arguments first, invasive disease may develop and second is shedding of *E histolytica* cysts in the environment is a public health concern.
- Asymptomatic *E dispar* infections should not be treated, but education should be pursued since it is a marker of fecal-oral contamination.
- Amebic colitis is first treated with a nitroimidazole derivative, followed by a luminal agent to eradicate colonization.

- Amebic liver abscess can be cured without drainage and even by one dose of metronidazole. Clinical defervescence should occur during the first 3-4 days of treatment. Metronidazole failure may be an indication for surgical intervention. Treatment with a luminal agent should also follow.
- Disseminated amebiasis should be treated with metronidazole, which can cross the brain-blood barrier.
- Empirical antibacterial agents should be used concomitantly if perforated viscus is a concern (Stanley SL, 2003).

1.15 Metrnidazole:

It is a nitroimidazole antibiotic medication used particularly for anaerobic bacteria and protozoa. Metronidazole, taken up by diffusion, is selectively absorbed by anaerobic bacteria and sensitive protozoa. Once taken up by anaerobes, it is non-enzymatically reduced by reacting with reduced ferredoxin, which is generated by pyruvate ferredoxin oxido-reductase. This reduction causes the production of toxic products to anaerobic cells, and allows for selective accumulation in anaerobes. The metronidazole metabolites are taken up into bacterial DNA, and form unstable molecules. This function only occurs when metronidazole is partially reduced, and because this reduction usually happens only in anaerobic cells.

Used in the Treatment:

- Acute intestinal amebiasis (amebic dysentery) and amebic liver abscess.
- Anaerobic infections, the intravenous form.
- Intra-abdominal infections, including peritonitis, intra-abdominal abscess, and liver abscess.

Side Effects:

- Nausea
- Diarrhea
- Metallic taste in the mouth
- Hypersensitivity reactions (rash, itch, flushing, fever)

- Dizziness
- Headache
- Vomiting
- Glossitis
- Tomatitis

1.16 Tinidazole

Used in the Treatment:

- Infections from amoebae, giardiasis and trichomonas.
- Treat a variety of other bacterial infections.

Side Effects:

- Metallic/bitter taste
- Nausea
- Anorexia
- Indigestion/cramps/belly discomfort
- Vomiting
- Constipation
- Weakness/fatigue/malaise
- Dizziness
- Headech

1.17 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.

1.18 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.

1.19Emetine

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.

1.20 Ornidazole:

Ornidazole is a drug that cures some protozoan infections.

Used in the Treatment:

- Anaerobic infections both pre & post operatively
- Bacterial vaginosis
- Amoebic dysentery
- Amoebic liver abscess
- Hepatic and intestinal amoebiasis
- Other protozoan infection like Giardiasis, Trichomoniasis

Side Effects:

- ❖ Abnormal metallic taste
- ❖ Diarrhea
- ❖ Drowsiness
- ❖ Nausea, vomiting
- ❖ Headache
- ❖ Sleep disturbances

1.21 Secnidazole:

- ❖ Congener with same spectrum of activity and potency.
  - o Oral absorption is rapid and complete.
  - o 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.

Treatement pattern:

- **Asymptomatic intestinal infection**
  - ✓ Luminal agent – Diloxanide furoate (500mg, x3daily for 10d)
  - ✓ Iodoquinol (650mg x3 daily for 21 d), or
  - ✓ Paromomycin sulfate (10mg/kg x3 daily for 7d)
- **Mild to moderate intestinal infection**
  - ✓ Metronidazole (750mg x3 daily for 10d OR 500mg IV every 6h for 10d), or
  - ✓ Tinidazole (2g daily for 3 d), PLUS
  - ✓ Luminal agent
- **Severe intestinal infection**
  - ✓ Metronidazole (750mg x3 daily for 10d OR 500mg IV every 6h for 10d), or
  - ✓ Tinidazole (2g daily for 3 d), PLUS
  - ✓ Luminal agent

- **Hepatic abscess, amoeba, other extraintestinal disease**
  - ✓ Metronidazole (750mg x3 daily for 10d OR 500mg IV every 6h for 10d), or
  - ✓ Tinidazole (2g daily for 5 d), PLUS
  - ✓ Luminal agents.

1.22 Nitazoxanite

Nitazoxanide, also known by the brand names Alinia and Annita (and by Daxon, Dexidex, Kidonax, Pacovanton and Paramix in Mexico, by Nitax, Zoxanide, Nitoxin, Zox, Nitazox and Toza in Bangladesh) is a synthetic nitrothiazolyl-salicylamide derivative and an antiprotozoal agent.

Nitazoxanide is a light yellow crystalline powder. It is poorly soluble in ethanol and practically insoluble in water (Rossignol JF, 2001).

1.22.1 History of Nitazoxanide

Nitazoxanide was originally discovered in the 1980s by Jean Francois Rossignol at the Pasteur Institute. Initial studies demonstrated activity versus tapeworms. *In vitro* studies demonstrated much broader activity. Dr. Rossignol co-founded Romark Laboratories, with the goal of bringing nitazoxanide to market as an anti-parasitic drug. Initial studies in the USA were conducted in collaboration with Unimed Pharmaceuticals, Inc. (Marietta, GA) and focused on development of the drug for treatment of cryptosporidiosis in AIDS. Controlled trials began shortly after the advent of effective anti-retroviral therapies. The trials were abandoned due to poor enrollment and the FDA rejected an application based on uncontrolled studies. Rather than abandon their efforts, Romark launched an impressive series of controlled trials. No other agent has proven efficacy in the treatment of cryptosporidiosis. However, a placebo controlled study of nitazoxanide in cryptosporidiosis demonstrated significant clinical improvement in adults and children with mild illness. Among malnourished children in Zambia with chronic cryptosporidiosis, a three-day course of therapy not only led to clinical and parasitologic improvement, but also improved survival. In Zambia and in a study conducted in Mexico, nitazoxanide was not successful in the treatment of cryptosporidiosis in advanced

infection with human immunodeficiency virus at the doses used. However, it was effective in patients with higher CD4 counts. Also, higher doses seem to have some effect in uncontrolled and unpublished studies. In treatment of giardiasis, nitazoxanide was superior to placebo and comparable to metronidazole. . However, a placebo controlled study of nitazoxanide in cryptosporidiosis demonstrated significant clinical improvement in adults and children with mild illness. Nitazoxanide was successful in the treatment of metronidazole-resistant giardiasis. Studies have suggested efficacy in the treatment of cyclosporiasis, isosporiasis, and amebiasis.

1.22.2 Mechanism of action

Nitazoxanide to be an inhibitor of pyruvate:ferredoxin oxidoreductase (PFOR) of *H. pylori*. PFOR catalyzes the oxidative decarboxylation of pyruvate to acetyl coenzyme A (acetyl-CoA) and CO_2, with reducing equivalents transferred to either ferredoxin or flavodoxin (Fig). These enzymes are also found in the amitochondriate eukaryotic human parasites (*Trichomonas vaginalis*, *Entamoeba histolytica*, and *Giardia intestinalis*), *Cryptosporidium parvum*, most anaerobic bacteria (*Clostridium* spp.), archaea, and microaerophiles of the epsilon proteobacterial group. Several MIC-based studies have shown NTZ to be very potent against large numbers of species of anaerobic bacteria, all of which utilize PFOR for the catabolism of pyruvate. Importantly, PFORs, which are highly conserved through evolution and in function , differ from the NADH-producing pyruvate dehydrogenase (PDH) complex that is exclusive to mammals and most eubacteria by their requirement for ferredoxin or flavodoxin as electron acceptors by tight binding of thiamine pyrophosphate (TPP) to the enzyme, and by the reversible nature of the enzyme in CO_2 fixation.

Here we show that NTZ is a noncompetitive inhibitor of the PFORs of luminal parasites, species of *Clostridium* including *C. difficile*, and the microaerophiles *Campylobacter jejuni* and *H. pylori*. Mechanistic studies with the PFOR of *H. pylori* indicate that NTZ inhibits an early step of the PFOR reaction, since the drug blocks the formation of CO_2 and acetyl-CoA, and the transfer of reducing equivalents to redox-active dyes. ^{1}H nuclear magnetic resonance (NMR) studies identified two apparent forms of NTZ: a biologically active anion (pK_a = 6.18) and a biologically inactive protonated form of NTZ at lower pH (HNTZ). While NTZ was less efficient in inhibiting the

pyruvate dehydrogenase activity of *Escherichia coli* in direct enzyme assays, bacterial growth was substantially inhibited in a glucose minimal medium, suggesting that the microbial spectrum of NTZ for non-PFOR-containing organisms might be influenced by nutrition. NTZ may be the first example of an antimicrobial agent that targets the "activated cofactor" of an enzymatic reaction rather than its substrate or catalytic sites, a potentially novel mechanism that may escape mutation-based drug resistance.

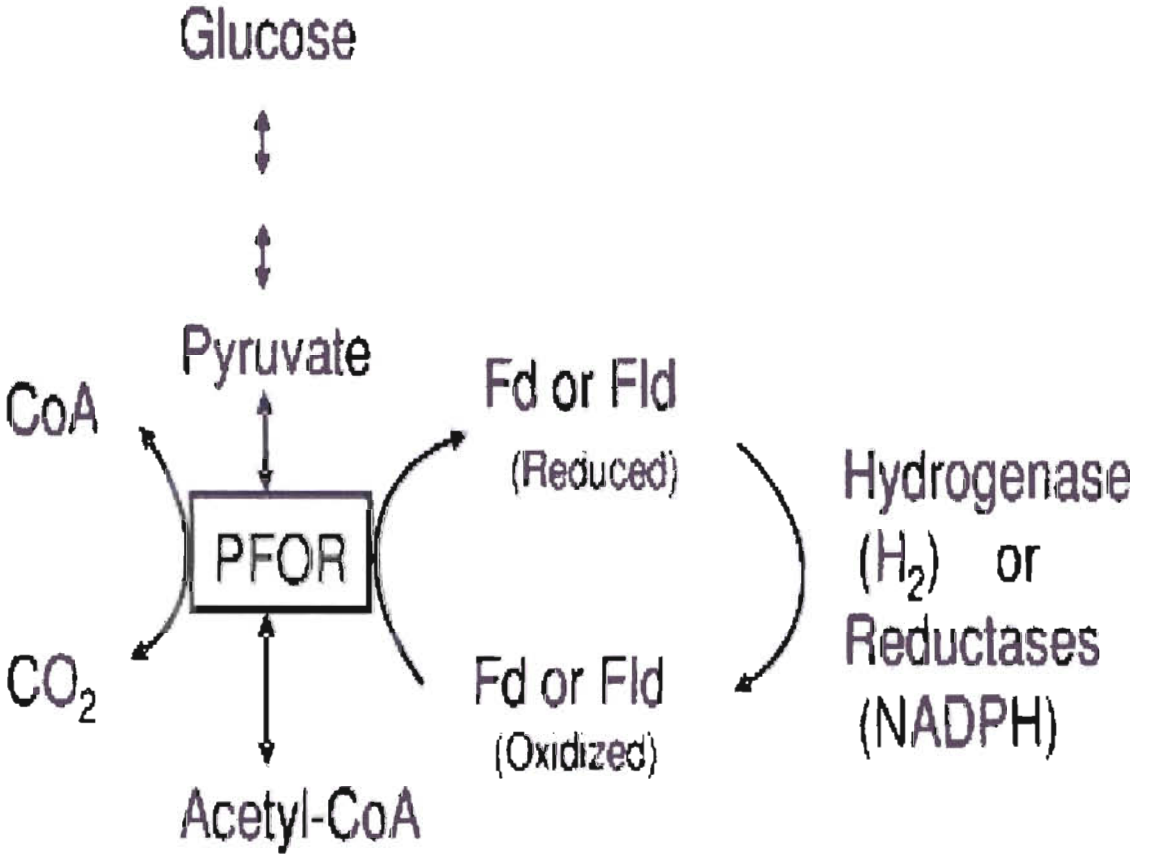


Figure 7: Mechanism of action of Nitazoxanide

1.22.3 Clinical pharmacology

Absorption:

Following oral administration of aniline tablets or Oral Suspension, maximum plasma concentrations of the active metabolites tizoxanide and tizoxanide glucuronide are observed within 1-4 hours. The parent nitazoxanide is not detected in plasma. Pharmacokinetic parameters of tizoxanide and tizoxanide glucuronide are shown in below:

Mean (Â±SD) plasma pharmacokinetic parameter values following administration of a single dose of one 500 mg Alinia Tablet with food to subjects >/=12 years of age

	Tizoxanide			**Tizoxanide glucuronide**		
Age	C_{max} (Âµg/mL)	T_{max} * (hr)	$AUC_{(tau)}$ (ÂµgÂ·hr/mL)	C_{max} (Âµg/mL)	T_{max} * (hr)	$AUC_{(tau)}$ (ÂµgÂ·hr/mL)
12-17 years	9.1 (6.1)	4.0 (1-4)	39.5 (24.2)	7.3 (1.9)	4.0 (2-8)	46.5 (18.2)
>/=18 years	10.6 (2.0)	3.0 (2-4)	41.9 (6.0)	10.5 (1.4)	4.5 (4-6)	63.0 (12.3)

* T_{max} is given as a Mean (Range)

Mean (Â± SD) plasma pharmacokinetic parameter values following administration of a single dose of Alinia for Oral Suspension with food to subjects 1 through 11 years of age

	Tizoxanide				Tizoxanide glucuronide		
Age	Dose	C_{max} (Âµg/mL)	T_{max} * (hr)	AUC_{inf} (ÂµgÂ·hr/mL)	C_{max} (Âµg/mL)	T_{max} * (hr)	AUC_{inf} (ÂµgÂ·hr/mL)
1-3 years	100 mg	3.11 (2.0)	3.5 (2-4)	11.7 (4.46)	3.64 (1.16)	4.0 (3-4)	19.0 (5.03)
4-11 years	200 mg	3.00 (0.99)	2.0 (1-4)	13.5 (3.3)	2.84 (0.97)	4.0 (2-4)	16.9 (5.00)

* T_{max} is given as a Mean (Range)

Alinia for Oral Suspension is not bioequivalent to Alinia Tablets. The relative bioavailability of the suspension compared to the tablet was 70%.

Effect of Food:

When Alinia Tablets are administered with food, the $AUC_{(tau)}$ of tizoxanide and tizoxanide

in plasma is increased almost two-fold and the C_{max} is increased by almost 50%.

When Alinia for Oral Suspension was administered with food, the $AUC_{(tau)}$ of tizoxanide and tizoxanide glucuronide increased by about 45-50% and the C_{max} increased by

Alinia Tablets and for Oral Suspension were administered with food in clinical trials and hence they are recommended to be administered with food (Musher D M. et, al. 2005).

Multiple dosing: Following oral administration of a single Alinia Tablet every 12 hours for 7 consecutive days, there was no significant accumulation of nitazoxanide metabolites tizoxanide or tizoxanide glucuronide detected in plasma.

Distribution: In plasma, more than 99% of tizoxanide is bound to proteins.

Metabolism: Following oral administration in humans, nitazoxanide is rapidly hydrolyzed to an active metabolite, tizoxanide (desacetyl-nitazoxanide). Tizoxanide then undergoes conjugation, primarily by glucuronidation. *In vitro* metabolism studies have demonstrated that tizoxanide has no significant inhibitory effect on cytochrome P450 enzymes.

Elimination: Tizoxanide is excreted in the urine, bile and feces, and tizoxanide glucuronide is excreted in urine and bile. Approximately two-thirds of the oral dose of nitazoxanide is excreted in the feces and one-third in the urine (Musher D M. et, al. 2005).

1.22.4 Pharmacokinetics

The objective of this study was to gather first information on the time course of plasma concentrations and urinary excretion of the antiprotozoal nitazoxanide (N) and to identify potential metabolites in healthy subjects after a single oral dose of 500 mg of nitazoxanide. The clinical trial was conducted as an open single oral dose study in 6 healthy male subjects. After a standardized continental breakfast the subjects took a single oral dose of 500 mg nitazoxanide (coated tablet) with 100 ml tap water. The plasma concentration and the urinary excretion of nitazoxanide (N), desacetyl-nitazoxanide (DN), aminonitrothiazole (ANT), acetylsalicylate (AS), salicylate (S), gentisate (G) and salicylurate (SU) were monitored up to 72 h after administration. The

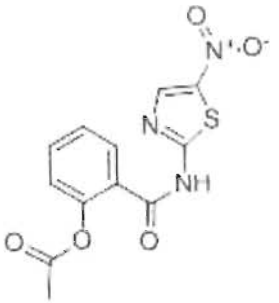
only measurable species in plasma was DN, which reached a Cmax of 1.9 mg/l (range 1.1-2.5) 2-6 h after dosing, and an AUC of 3.9-11.3 mg x h/l. Its terminal half-life ranged from 1.03 to 1.6 h. DN was extensively bound to plasma proteins (> 97.5%). Only 8% of the dose was recovered in the urine, in the form of DN (5%), SU (3%), and traces of ANT (0.1%). In vitro N was very rapidly hydrolyzed to DN by plasma esterase's (Musher D M. et, al. 2005).

1.22.5 Dosage forms:

Nitazoxanide is available in two oral dosage forms - tablet (500 mg) and oral suspension (100 mg per 5 ml when reconstituted).

1.22.6 Details of Nitazoxanide:

Nitazoxanide Chemical Properties



Chemical Name: Nitazoxanide

Synonyms: 2-(acetolyloxy)-n-(5-nitro-2-thiazolyl)benzamide;2-(acetyloxy)-n-(5-nitro-2-thiazolyl)-benzamid;n-(5-nitro-2-thiazolyl) salicylamidacetate(ester);n-(5-nitro-2-thiazolyl)salicylamideacetate(ester);AURORA KA-645;o-[n-(5-nitrothiazol-2-yl)carbamoyl]phenyl acetate;NITAZOXANIDE;nitazoxamide;PH-5776, Cryptaz, 2-(Acetyloxy)-N-(5-nitro-2-thiazolyl)benzamide;2-(Acetyloxy)-N-(5-nitro-2-thiazolyl)benzamide;[2-[(5-nitro-1,3-thiazol-2-

yl)carbamoyl]phenyl] acetate;[2-[(5-nitro-1,3-thiazol-2-yl)carbamoyl]phenyl]ethanoate;acetic acid [2-[(5-nitrothiazol-2-yl)carbamoyl]phenyl] ester.

CBNumber: CB7219063

MolecularFormula: C12H9N3O5S

1.22.7 Uses

Nitazoxanide is a first-line choice for the treatment of illness caused by *Cryptosporidium parvum* or *Giardia lamblia* infection in immunocompetent adults and children, and is an option to be considered in the treatment of illness caused by other protozoa and/or helminths. It is used for the treatment of infectious diarrhea caused by *Cryptosporidium parvum* and *Giardia lamblia* in patients 1 year of age and older. Nitazoxanide is currently in Phase II clinical trials for the treatment of hepatitis C, in combination with peginterferon alfa-2a and ribavirin (Rossignol JF. et, al. 2001).

Chronic hepatitis B

Nitazoxanide alone has shown preliminary evidence of efficacy in the treatment of chronic hepatitis B over a one year course of therapy. Nitazoxanide 500 mg twice daily resulted in a decrease in serum HBV DNA in all of 4 HBeAg-positive patients, with undetectable HBV DNA in 2 of 4 patients, loss of HBeAg in 3 patients, and loss of HBsAg in one patient. Seven of 8 HBeAg-negative patients treated with nitazoxanide 500 mg twice daily had undetectable HBV DNA and 2 had loss of HBsAg. Additionally, nitazoxanide monotherapy in one case and nitazoxanide plus adefovir in another case resulted in undetectable HBV DNA, loss of HBeAg and loss of HBsAg. These preliminary studies showed a higher rate of HBsAg loss than any currently licensed therapy for chronic hepatitis B.The similar mechanism of action of interferon and nitazoxanide suggest that stand-alone nitazoxanide therapy or nitazoxanide in concert with nucleos(t)ide analogs have the potential to increase loss of HBsAg, which is the ultimate end-point of therapy (Korba BE, et, al. 2008).

Chronic hepatitis C

Romark made the decision to initially focus on the potential treatment of chronic hepatitis C with nitazoxanide. Three phase II studies of nitazoxanide for the treatment of chronic hepatitis C have been completed and communicated in publications or presentations at national and international meeting. The first study was a randomized, double-blind, placebo-controlled study of the treatment of chronic hepatitis C with nitazoxanide 500 mg twice daily in 50 adult patients with chronic hepatitis C infected with genotype 4 (Korba BE, et, al. 2008).

1.22.8 Side Effects:

Stop taking nitazoxanide and seek emergency medical attention if experience a rare allergic reaction (difficulty breathing; closing of the throat; swelling of the lips, tongue, or face; or hives). Other, less serious side effects may be more likely to occur.

These side effects have been reported in patients 12 years of age or older receiving nitazoxanide:

- abdominal pain
- diarrhea
- headache
- nausea
- greenish tint of urine

Similar side effects are seen in children between 1 year and 12 years of age, except that some children experience vomiting. Usually the side effects are mild and transient (Rossignol J F. et al 2006).

1.22.9 Nitazoxanide Suspension

All medicines may cause side effects, but many people have no, or minor, side effects. When nitazoxanide suspension of severe allergic reactions :

- (Rash; Hives; Difficulty breathing; Tightness in the chest; Swelling of the mouth, Face, Lips, or Tongue);
- Bone pain;
- Fainting;
- Fast heartbeat;
- Severe or persistent dizziness;
- Shortness of breath;

1.22.10 Adverse effects

Side effects are mostly gastrointestinal, and include abdominal pain (6%), vomiting (1%), headache (3%), nausea (3%) and diarrhea (4%).In the clinical trials no serious adverse events were reported. No serious adverse events were reported in the prescribing information of the parent company.

1.22.11 Clinical Trials

Nitazoxanide has not been proven effective for treating diarrhea caused by Cryptosporidium parvum in patients with HIV/AIDS or in patients with a suppressed immune system.

A study performed in Egypt evaluated the effectiveness of nitazoxanide in individuals 12 years of age or older. Patients with diarrhea caused by *Cryptosporidium* were given nitazoxanide in syrup (25 ml) or tablet (500 mg) or a placebo for three days. Four days after stopping treatment, 96% of patients given the nitazoxanide tablets and 87% given the syrup improved. The parasite was cleared from 93% of patients treated with nitazoxanide tablets and 90% of those who were given the syrup. Another study in Egypt included patients 1-11 years of age with the adolescents and adults. After three days of treatment with nitazoxanide, diarrhea resolved in 80% of patients, whereas diarrhea resolved in 41% of patients given placebo (Rossignol J F. et, al. 2006).

1.22.12Warning & Precautions

Nitazoxanide has been assigned to pregnancy category B by the FDA. Animal studies have failed to reveal evidence of fetal harm. There are no controlled data in human pregnancy. Nitazoxanide is only recommended for use during pregnancy when benefit outweighs risk. There are no data on the excretion of nitazoxanide into human milk. The manufacturer recommends that caution be used when administering nitazoxanide to nursing women. The pharmacokinetics of nitazoxanide in patients with compromised renal or hepatic function has not been studied. Therefore, nitazoxanide must be administered with caution to patients with hepatic and biliary disease, to patients with renal disease and to patients with combined renal and hepatic disease.

1.22.13 Drug Interaction

Tizoxanide, the active metabolite of nitazoxanide is highly bound to plasma protein (>99.9%). Therefore, caution should be used when administering nitazoxanide concurrently with other highly plasma protein-bound drugs with narrow therapeutic index. as competition for binding sites may occur (e.g., warfarin).

1.22.14 Doses

Nitazoxanide is available as a tablet or as a syrup. The tablets are 500 mg. The strawberry-flavored syrup contains 100 mg nitazoxanide in 5 ml of suspension. Tablets are typically given to adolescents (12 years of age or older) and adults, whereas children between 1 and 12 years old are given the syrup.

Each dose of nitazoxanide should be taken with food. The recommended dosage regimens are as follows:

- Children 1-3 years of age: 5 ml syrup every 12 hours for 3 days
- Children 4-11 years of age: 10 ml of syrup every 12 hours for 3 days
- Adolescents over 12 years of age and adults: 500 mg tablet or 25 ml of syrup every 12 hours for 3 days.

CHAPTER 2:

THE AIM & SIGNIFICANCE OF THE STUDY

2.1 Aim of the study

Bangladesh is among third world countries and a big percentage of its population cannot afford expensive medication. *Entamoeba histolytica* and it infects approximately 10% of the world's population. Bangladesh is not beyond this threat and mostly resident of urban areas of the country face troubles to deal with amebiasis. So amebiasis is a common disease in the entire world. 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 (Walsh, 1998).

In past many research work has been done by the researchers. They find out the efficacy of herbal product and synthetic product to treat this disease and most of the sources has showed anti-amoebic properties satisfactory. Nitazoxanide is one of the anti protozoal drug. . It is a synthetic nitrothiazolyl-salicylamide derivative and an antiprotozoal agent. Nitazoxanide is a prodrug, following oral administration; it is rapidly hydrolyzed to its active metabolite, tizoxanide which is 99% protein bound and peak concentrations are observed 1–4 hours after administration. It is excreted in the urine, bile and feces. It is less costly.

Therefore, the present study was aimed to find out the *in vitro* sensitivity of nitazoxanide(Zoxanide) tablets from Bangladeshi pharmaceuticals against clinical isolates of *E. histolytica.*

2.2 Significance of the study

Most of the people in Bangladesh have not enough money to bear the expenses of their medication. Proper medication is another problem in Bangladesh. People eat very unhygienic food and water. Amebiasis mainly caused by the protozoan *E. histolytica.* Amoebiasis is a common disease and can be spread through water easily. Unlike any developed country, we are struggling to find an efficient and economical drug to cure Amoebiasis completely.

In this situation a cost effective anti amoebic drug is very much essential for the treatment of amoebiasis. Zoxanide tablet can be the right choice for the people. However it is a safe drug to use against amoebiasis.

This research paper shows that Nitazoxanite is very effective in the treatment of amoebiasis. The brand (Zoxanide) from SILVA Pharmaceutical Company, compared with the standard Nitaxoanide where the brand gives almost same result as like as standard.

Therefore, the main significance of this study is to know about the efficacy and safeties of using the drug Zoxanide. Because of the 100% inhibitory action of Zoxanide in the concentration of 4.6 μM, the logical explanation is that, Zoxanide is an innovative treatment option against amoebiasis. Mass people can use this drug over other conventional anti amoebic drug.

CHAPTER 3:

Material and methods

3. Materials and Methods

3.1 Research design

The research design in the "*In vitro* Sensitivity test of Zoxanide againest clinical isolation of *Entamoeba histolytica*"

3.2 Clinical isolation and collection

Isolation is usually achieved by growing the species in an environment that was previously sterilized and was thereby rid of contaminating organisms. 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 (Robinson GL, 1968).

3.3 Instruments

- Microscope
- Haemocytometer
- Microtip
- Analytical Balance
- Morter and pastle
- Vortex machine
- Micropipettes
- Eppendrof (1ml and 2ml)
- Microtiter plate

3.4 Preparation of culture media

Clinical isolates are cultured in first xenic media then it is axenically process to axenic or pure culture. Mentioned below these are the processes:

3.4.1 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. And this is currently the most widely used medium for axenic cultivation of *E. histolytica* (Gillin, et, al. 1978).

3.4.2 Xenic Culture Media

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 added. Then again heat after the heat the solution must be cool and taken into a small glass bottle by a syringe .Then placed in the auto clave, after completed the auto clave, the liquid solution bottle taken into a box and the box must be settled by an angle state because the angle bottle can be suitably for parasite (Jean-Francois, et. al, 2010).

3.4.3 Preparation of Rice Starch:

Purified rice starch is important for growth of *E. histolytic* in all the following media. To prepare (18), 500 mg of powdered rice starch is placed into each of several culture tubes (16 by 125 mm) and is heated at 150°C, with loose caps, in a dry oven for about 3 hours. Sterilization of the rice starch prevents alteration of the bacterial flora when it is added to the culture and is thus recommended.

After cooling, the caps are tighten and are stored at room temperature. To prepare for use, 9.5 ml of sterile distilled water or phosphate-buffered saline (PBS) is added to one tube. The tubes are vortexed for resuspension. 1 ml of the resuspended starch is distributed to each of 10 tubes containing 9 ml of sterile water or PBS, and they are refrigerated.

The final concentration of diluted rice starch is 5 mg/ml. Before use, the rice is resuspend by vortexing or vigorous shaking. The desired volume is taken into culture tubes with medium, making sure that the stock rice stays in suspension. Different isolates require

various amounts of rice starch, but 0.2 ml (1 mg) 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.

3.4.4 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.

3.4.5 Monophasic culture media

3.4.5.1 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).

3.4.5.2 LYSGM

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

3.4.6 Axenie Culture Media

One constant problem facing those who rely on axenic cultures is the fastidiousness of these organisms. Although the others are also affected to a significant degree, this is especially true of *E. hisiolytica.* Lot-to-lot variations in several components of the axenic culture media in particular can have profound effects on the ability of a medium to support growth of the organisms; some lots may even be toxic. Trypticase (casein digest peptone), yeast extract, and serum are the medium components most commonly affected, but the quality of the distilled water and even the type of glass used in making the culture tubes can cause problems (screw-cap borosilicate glass tubes should be used when possible). For this reason, we highly recommend that those wishing to undertake axenic cultivation of these organisms test the ability of each new lot of reagent to support growth before starting to use it.

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

3.5.1 *E. histafytica.* LYI-S-2:

In the course of developing LYI-S, several combinations of liver digest and yeast extract were studied. One of these, designated LYI-S-2 (containing liver digest, yeast extract, iron, and serum), was 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, YI-S, was extensively tested and published. No difference in the ability of YI-S and LYI-S-2 to support growth of *E. histolytica* was observed (unpublished results). After publication of YI-S, further testing within our laboratory and by others disclosed the fact that some lots of yeast extract would not support any growth of the ameba while with others growth was very poor. In the case of the latter it was found that substitution of a small amount of liver digest for an equal amount of yeast extract enhanced growth considerably. LYI-S-2 is

recommended when a given lot of yeast extract will support some growth, though poorly, of *E. histolytica*. LYI-S-2 is identical to YI-S except that weight for weight it contains 0.5% neutralized liver digest and only 2.5% yeast extract. It has been used in the long-term cultivation of several isolates of *E. histolytica* and a number of other *Entamoeba* species, with yields similar to those observed with the more widely used TYI-S-33 and YI-S.

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

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 %

3.5.2 Establishment of Cultures

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

3.6 General considerations:

E. histolytica needs to be established in xenic culture. The most-common source of material will be stool samples, and this is what is assumed below. In rare instances rectal biopsy specimens 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 amebae into xenic culture. Such material has also been used for the direct establishment of *E. histolytica* into monoxenic cultures with either a bacterium or a trypanosomatid as the associate. Unless a stool sample is from a patient with dysentery, it is likely that the amebae will be in the encysted form. This allows for several approaches to the establishment of cultures (Dobell C. et, al. 1926).

3.7 Elimination of unwanted organisms:

One of the banes of xenic cultivation is the likelihood of unwanted organisms overgrowing the desired ameba. The most-frequent source of this problem is *B. 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.komims* with acrifiavin as first described by Dobell and Laidlaw, 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.

The first method also was described by Dobeil and Laidlaw in 1926 . In this method, cysts are treated with 0.1 N hydrochloric acid at room temperature for 10 min, washed thoroughly with distilled water, and reinoculated 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 nonencysted amebae while leaving the cysts intact and viable. We have found that it is not necessary in most cases for the cysts to be mature. We do not know whether the cysts complete their maturation upon inoculation or whether immature cysts respond to the stimulus and excyst directly. The cysts used can be either from stool or cultures; *Entamoeba* cultures in LE medium m particular frequently produce small numbers of cysts spontaneously. 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 (Dobell C. et, al. 1926).

The successful separation of the flora can be checked by inoculating a substantial volume into fresh medium with rice starch and checking for amebal growth. The flora can be stored at 4°C indefinitely. The second method is- that of Smedley and is- used when *B. hominis* appears in cultures after inoculation. 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 min.

The material is then repelleted and inoculated into fresh culture medium. Perhaps surprisingly, many *Entamoeba* trophozoites survive this treatment while *B. hominis* generally does not. A few cells or cysts of *B. hominis* may survive and start to grow, and the procedure will then need to be repeated.

The advantages of Smedley's method are 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. However, occasional instances of balanced mixed cultures are known (Smedley S.R, 1956).

3.8 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

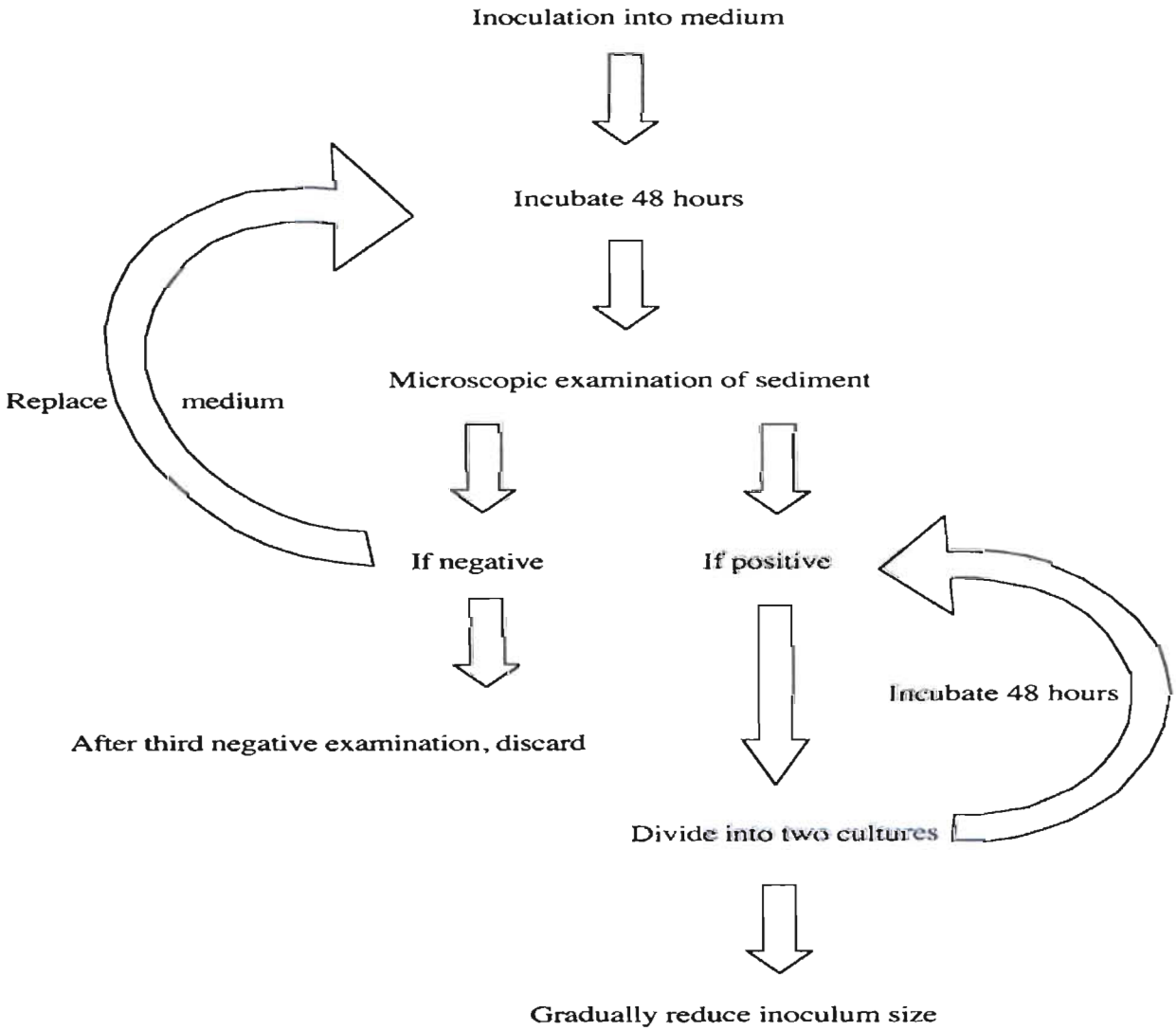


Figure 8: Flow diagrams illustrating the stages in establishing luminal protists in culture.

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).

3.9 Principles of inducing encystment of *E. histolytic*

The methods of inducing encystment of *E. histofytica* 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. Each point these authors make holds true to this day, and anyone attempting to induce encystment must bear these points in mind at all times. To date, cysts of this species have been induced only in xenic culture.

No one as far as we can determine has published a detailed account of the process of inducing encystment. Here we present a protocol used for many years in the NIH Laboratory of Parasitic Diseases but never previously published in detail. Three things are of special concern in obtaining cysts: 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. Here again some are better than others. 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).

3.10 Protocol of encystment

Day 1

Begin the process 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, 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. 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.

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. Incubate 48 h.

Day 6.

Harvest the 12 cultures and subculture as on day 4. Incubate the 24 cultures for 48 h.

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. Collect the sediments 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 hr

Day 10.

Remove a small drop of sediment from each culture, stain with Lugo Fs 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, incubate an additional 24 hrs.

3.11 Protocol: excystment of cysts induced *in vitro*:

Inducing *E. histotyticct* to excyst is relatively easy compared to getting the ameba to encyst. How this is accomplished depends on the goal. 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. While the amoebae will excyst in the axenic media, no one, as yet, has been able get them to encyst m this environment. 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 as recommended above. 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 excy station will have done so by the end of *6* h.

3.12 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 well in our hands.

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

(ii). Chill 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 105, 2 x 105, and x 105 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). Subculture. 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 (do not add additional bacteria after the initial inoculation of the medium). Later, as amebic growth improves, transfer smaller portions, e.g., one-third to one-four.

3.13 Maintenance of cultures

Sunday-Tuesday-Thursday 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 inoculate or the amount of rice and antibiotics to be added to the medium for optimal growth, ft 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. Established axerric cultures of *E. htstofytica* are passaged at 72- and 96-h intervals, with a Sunday-Thursday schedule being convenient. Visual inspection of every culture before subculture is recommended, since what appears to be a heavy culture may in fact contain many lysed cells, indicating that the inoculum previously used was too large. An increased inoculum volume may be warranted for the subsequent subculture to compensate for the dead amoebae. Likewise, parallel duplicate cultures are recommended in case of inadvertent contamination or tube breakage. The unused culture can be kept at 33°C as a backup in case of problems. The method for subculturing many types of cultures is essentially the same. Cultures are chilled in an ice-water bath for 5 min (xenic cultures and axenic *E. histolytica)* 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 containing fresh medium. The tubes are capped tightly and incubated at 36 to 37°C, either vertically at 5° to the horizontal (established axenic cultures of *E. histolyticd).*

3.14 Preparation of Antiamebic agent

Nitazoxanide drug is a standard used in the study was collected as pure salt from SQUARE Pharmacuticals LTD, Dhaka, Bangladesh and aslo some other diffrent brand standard Nitazoxanide was weighed and dissolved in 1mL of Di methyl sulfoxide (DMSO). In a refrigerator the stock solution was stored.

3.15 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 was 4.6, 2.3, 1.15, 0.575, 0.287, 0.1437 and 0.0718 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 24 hrs the plate was taken from the incubator.

CHAPTER 4:

Result

4. Result

4.1 Measurement of amoebicidal activity:

The main target of this study is to see the amoebicidal activity of the drug nitazoxanide (ZOXANIDE). For this purpose The clinical isolates of *Entamoeba histolytica* were treated with Nitazoxanide 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 Nitazoxanide 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.

4.2 Trial- 1

4.2.1 Viable count of *E.histolytica* from standard & commercial product of Nitazoxanide (Zoxanide) after 24 hours incubation.

At trial 1 the initial count were **1.14×10^6 mL^{-1}.** After 24 hours the parasite counts of the control were **$1{\cdot}42 \times 10^6$ mL^{-1}**. The number of parasites are increased in the control after 24 hours incubation.

Table 1. Viable count of *E. histolytica* from Standard & Brand (Zoxanide) after 24 hours incubation.

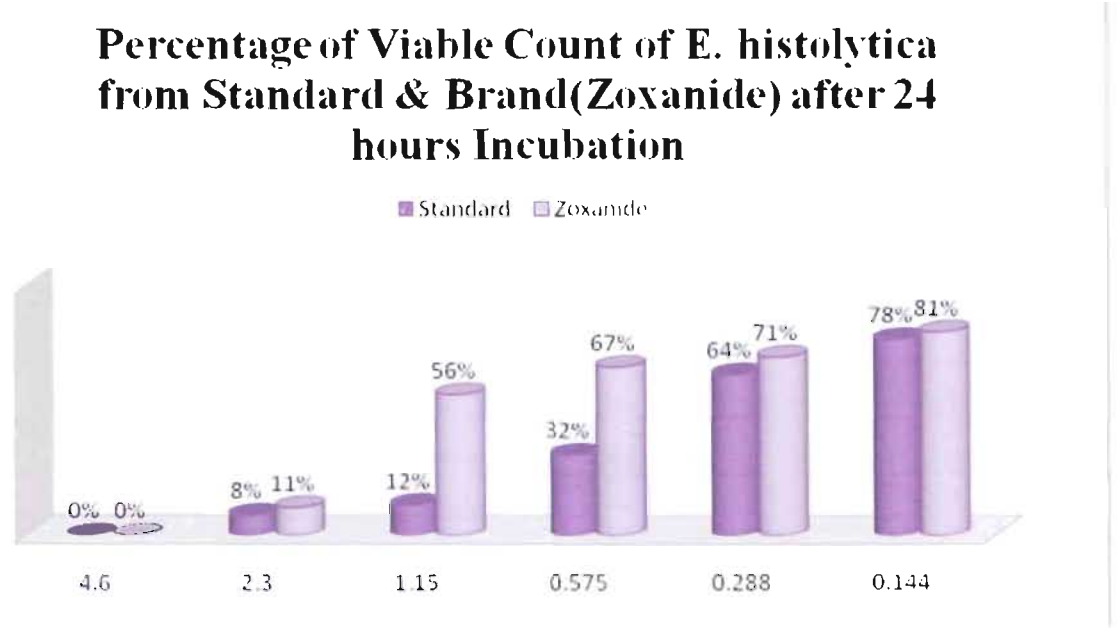
Concentration of Nitazoxanide (µM) Standard	Viable count of *E. histolytica*	Concentration of Nitazoxanide (µM) Drug(Zoxanide)	Viable count *E. histolytica*
4.6	$0{\cdot}00 \times 10^6$ mL^{-1}	4.6	$0{\cdot}00 \times 10^6$ mL^{-1}
2.3	$0{\cdot}087 \times 10^6 mL^{-1}$	2.3	$0{\cdot}13 \times 10^6 mL^{-1}$
1.15	$0{\cdot}14 \times 10^6$ mL^{-1}	1.15	$0{\cdot}64 \times 10^6$ mL^{-1}
0.58	$0{\cdot}37 \times 10^6 mL^{-1}$	0.58	$0.76 \times 10^6 mL^{-1}$
0.29	$0.73 \times 10^6 mL^{-1}$	0.29	$0.81 \times 10^6 mL^{-1}$
0.14	$0.89 \times 10^6 mL^{-1}$	0.14	$0.92 \times 10^6 mL^{-1}$

- It has been seen that after 24 hours incubation the viable count of *Entamoeba histolytica* were 0.89×10^6 mL^{-1}& 0.92×10^6 mL^{-1} for standard & brand respectively when the concentration of Nitazoxanide was 0·14 µM.
- The viable count of *Entamoeba histolytica* were 0.73×10^6 mL^{-1}, $0{\cdot}37\times10^6$ mL^{-1} , $0{\cdot}14\times10^6$ mL^{-1} and $0{\cdot}087\times10^6$ mL^{-1} when the concentration were 0.287, 0.575, 1.15 and 2.3 µM respectively, when treated with the standard preparation. At the same time the viable count from brand (Zoxanide) of *Entamoeba histolytica* were 0.81×10^6 mL^{-1}, 0.76×10^6 mL^{-1} , $0{\cdot}64\times10^6$ mL^{-1} and $0{\cdot}13\times10^6$ mL^{-1} when the concentration were 0.287, 0.575, 1.15 and 2.3 µM respectively, when treated with the Zoxanide.
- The viable count of *Entamoeba histolytica* was decreased to $0{\cdot}00\times10^6$ mL^{-1} & $0{\cdot}00\times10^6$ mL^{-1} when the concentration of Nitazoxanide was increased to 4.6µM for standard and brand(Zoxanide) respectively.

Table 2: Percentage of viable count

Concentration of Nitazoxanide (µM) Standard	Percentage of viable count	Concentration of Nitazoxanide (µM) Drug(Zoxanide)	Percentage of viable count
4.6	0%	4.6	0%
2.3	8%	2.3	11%
1.15	12%	1.15	56%
0.575	32%	0.575	67%
0.288	64%	0.288	71%
0.144	78%	0.144	81%

Graph 1. Percentage of viable count of *E. histolytica* from standard & commercial product (Zoxanide) of Nitazoxanide after 24 hours incubation.



4.2.2 Non-viable count of *E.histolytica* from standard & commercial product of Nitazoxanide (Zoxanide) after 24 hours incubation.

The initial counts of the parasite were **1.14×10^6 mL^{-1}**. After 24 hours the parasite counts of the control were **$1{\cdot}42\times10^6$ mL^{-1}**. The number of parasites are increased in the control 24 hours incubation.

Table 3: Non viable count *E. histolytica* from Standard & Brand (Zoxanide) after 24 hours incubation.

Concentration of Nitazoxanide (µM) Standard	Non Viable count of *E. histolytica*	Concentration of Nitazoxanide (µM) Drug(Zoxanide)	Non Viable count of *E. histolytica*
4.6	1.14×10^6 mL^{-1}	4.6	1.14×10^6 mL^{-1}
2.3	$1.05\times10^6 mL^{-1}$	2.3	$1.01\times10^6 mL^{-1}$
1.15	1.0×10^6 mL^{-1}	1.15	0.50×10^6 mL^{-1}
0.575	0.77×10^6 mL^{-1}	0.575	0.38×10^6 mL^{-1}
0.288	$0.71\times10^6 mL^{-1}$	0.288	$0.33\times10^6 mL^{-1}$
0.144	0.25×10^6 mL^{-1}	0.144	$0{\cdot}22\times10^6$ mL^{-1}

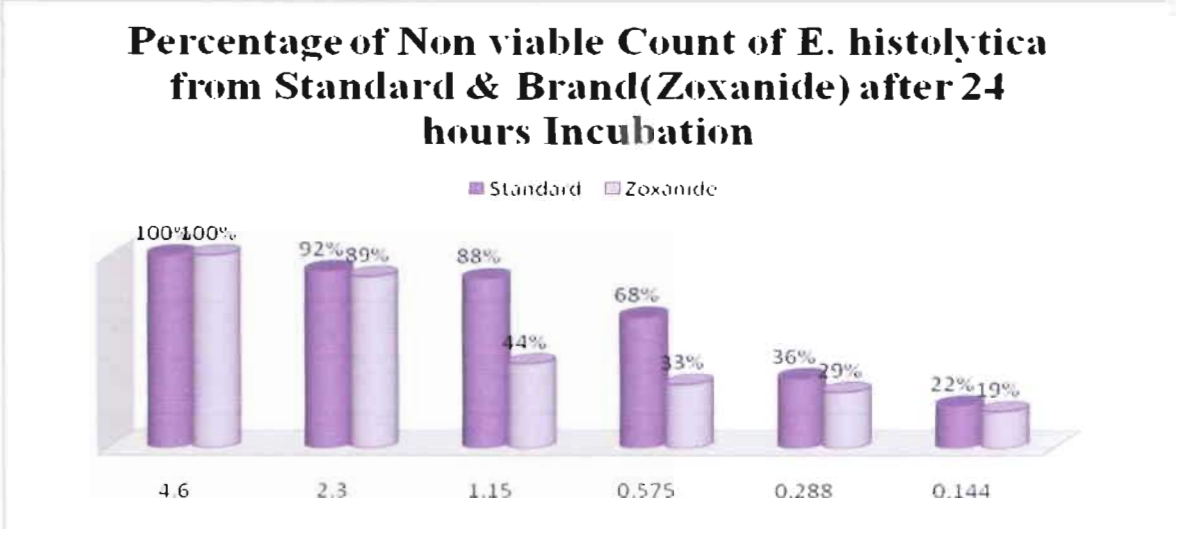
- After 24 hours incubation the non viable count of *Entamoeba histolytica* were 0.25×10^6 mL^{-1} and $0{\cdot}22\times10^6$ $mL^{-1}$when the concentration of Nitazoxanide was 0.144 µM for standard & brand respectively.

- In case of standard the non viable count of *E. histolytica* were 0.71×10^6 mL^{-1}, 0.77×10^6 mL^{-1}, 1.0×10^6 mL^{-1} and 1.05×10^6 mL^{-1} when the concentration were 0.29, 0.58, 1.15 and 2.3 µM respectively. At the same time the non viable count from brand (Zoxanide) of *Entamoeba histolytica* were $0.33\times10^6mL^{-1}$, 0.38×10^6 mL^{-1} , 0.50×10^6 mL^{-1} and 1.01×10^6 mL^{-1} when the concentration were 0.287, 0.575, 1.15 and 2.3 µM respectively.
- The non viable count of *Entamoeba histolytica* was increase to 1.14×10^6 mL^{-1} & 1.14×10^6 mL^{-1} when the concentration of Nitazoxanide was increased to 4.6µM for standard and brand respectively.

Table 4: Percentage of the non viable

Concentration of Nitazoxanide (µM) Standard	Percentage of the non viable count	Concentration of Nitazoxanide (µM) Drug(Zoxanide)	Percentage of the non viable count
4.6	100%	4.6	100%
2.3	92%	2.3	89%
1.15	88%	1.15	44%
0.575	68%	0.575	33%
0.288	36%	0.288	29%
0.144	22%	0.144	19%

Graph 2: Percentage of non viable count of *E. histolytica* fron standard & brand after 24 hours Incubation.



4.3 Trial-2

4.3.1 Viable count of *E.histolytica* from standard & commercial product of Nitazoxanide (Zoxanide) after 24 hours incubation.

At trial 2 the initial counts of the parasite were **1.14×10^6 mL^{-1}**. After 24 hours the parasite counts of the control were **1.38×10^6 mL^{-1}**. The number of parasites are increased in the control after 24 hours incubation.

Table 5. Viable count of *E. histolytica* for Standard & commercial product (Zoxanide) after 24 hours incubation.

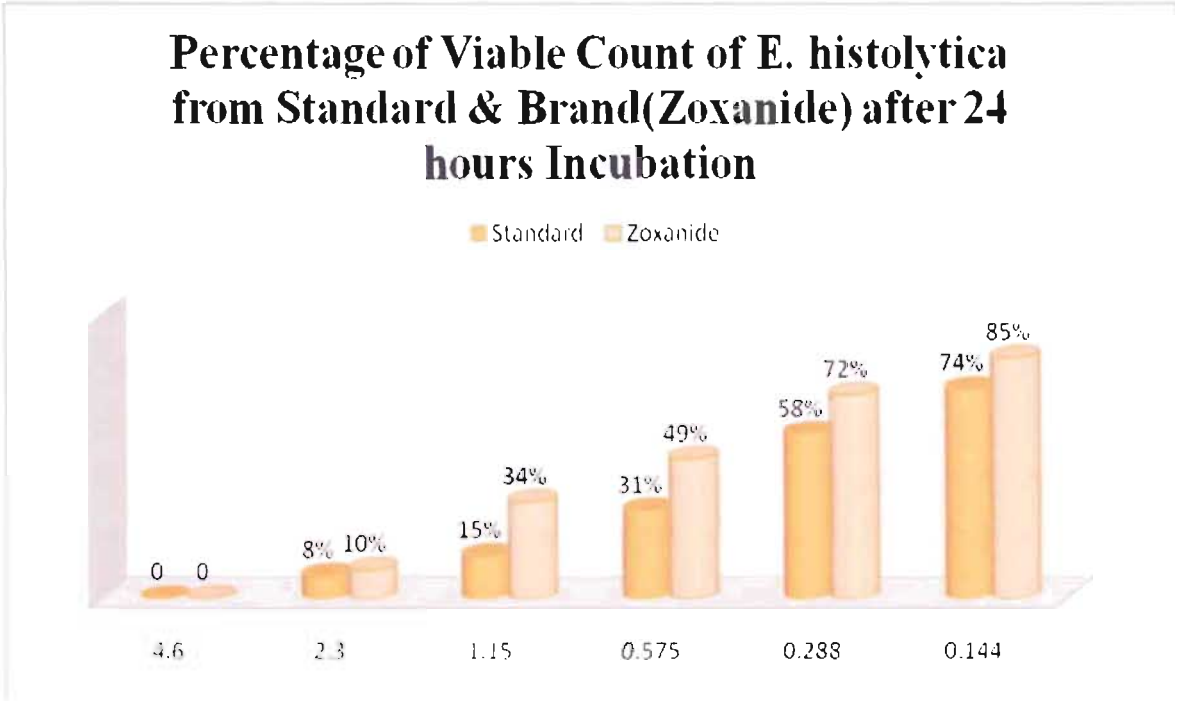
Concentration of Nitazoxanide (µM) Standard	Viable count of *E. histolytica*	Concentration of Nitazoxanide (µM) Drug(Zoxanide)	Viable count *E. histolytica*
4.6	0.00×10^6 mL^{-1}	4.6	$0{\cdot}00\times10^6$ mL^{-1}
2.3	$0{\cdot}09\times10^6$ mL^{-1}	2.3	$0{\cdot}12\times10^6$ mL^{-1}
1.15	0.17×10^6 mL^{-1}	1.15	$0{\cdot}39\times10^6$ mL^{-1}
0.575	0.36×10^6 mL^{-1}	0.575	0.56×10^6 mL^{-1}
0.288	0.66×10^6 mL^{-1}	0.288	0.82×10^6 mL^{-1}
0.144	0.84×10^6 mL^{-1}	0.144	0.98×10^6 mL^{-1}

- After 24 hours incubation the viable count of *Entamoeba histolytica* were 0.84×10^6 mL^{-1} & 0.98×10^6 mL^{-1} for standard & brand respectively when the concentration of Nitazoxanide was 0·144 µM.
- When treated with the standard preparation the viable count of *Entamoeba histolytica* were 0.66×10^6 mL^{-1}, 0.36×10^6 mL^{-1} , 0.17×10^6 mL^{-1} and $0{\cdot}09\times10^6$ mL^{-1} when the concentration were 0.287, 0.575, 1.15 and 2.3 µM respectively. At the same time the viable count from brand (Zoxanide) of *Entamoeba histolytica* were 0.82×10^6 mL^{-1}, 0.56×10^6 mL^{-1} , $0{\cdot}39\times10^6$ $mL^{-1}$and $0{\cdot}12\times10^6$ $mL^{-1}$when the concentration were 0.287, 0.575, 1.15 and 2.3 µM respectively, when treated with the Zoxanide.
- The viable count of *Entamoeba histolytica* was decreased to 0.00×10^6 mL^{-1} & $0{\cdot}00\times10^6$ $mL^{-1}$when the concentration of Nitazoxanide was increased to 4.6µM for standard and Zoxanide respectively.

Table 6: Percentage of the viable count

Concentration of Nitazoxanide (µM) Standard	Percentage of viable count	Concentration of Nitazoxanide (µM) Drug(Zoxanide)	Percentage of viable count
4.6	0	4.6	0
2.3	8%	2.3	10%
1.15	15%	1.15	34%
0.575	31%	0.575	49%
0.288	58%	0.288	72%
0.144	74%	0.144	85%

Graph 3: Percentage of viable count of *E. histolytica* fron standard & brand after 24 hours Incubation.



4.3.2 Non-viable count of *E.histolytica* from standard & commercial product of Nitazoxanide (Zoxanide) after 24 hours incubation.

Table 7: Non viable count of *E. histolytica* from Standard & commercial product (Zoxanide) after 24 hours incubation.

Concentration of Nitazoxanide (µM) Standard	Non Viable count of *E. histolytica*	Concentration of Nitazoxanide (µM) Drug(Zoxanide)	Non Viable count of *E. histolytica*
4.6	$1.14\times10^{6}mL^{-1}$	4.6	$1.14\times10^{6}mL^{-1}$
2.3	$1.05\times10^{6}mL^{-1}$	2.3	$1.02\times10^{6}mL^{-1}$
1.15	$0.97\times10^{6}mL^{-1}$	1.15	$0.75\times10^{6}mL^{-1}$
0.58	$0.78\times10^{6}mL^{-1}$	0.58	$0.58\times10^{6}mL^{-1}$
0.29	$0.48\times10^{6}mL^{-1}$	0.29	$0.32\times10^{6}mL^{-1}$
0.14	$0.30\times10^{6}mL^{-1}$	0.14	$0{\cdot}16\times10^{6}mL^{-1}$

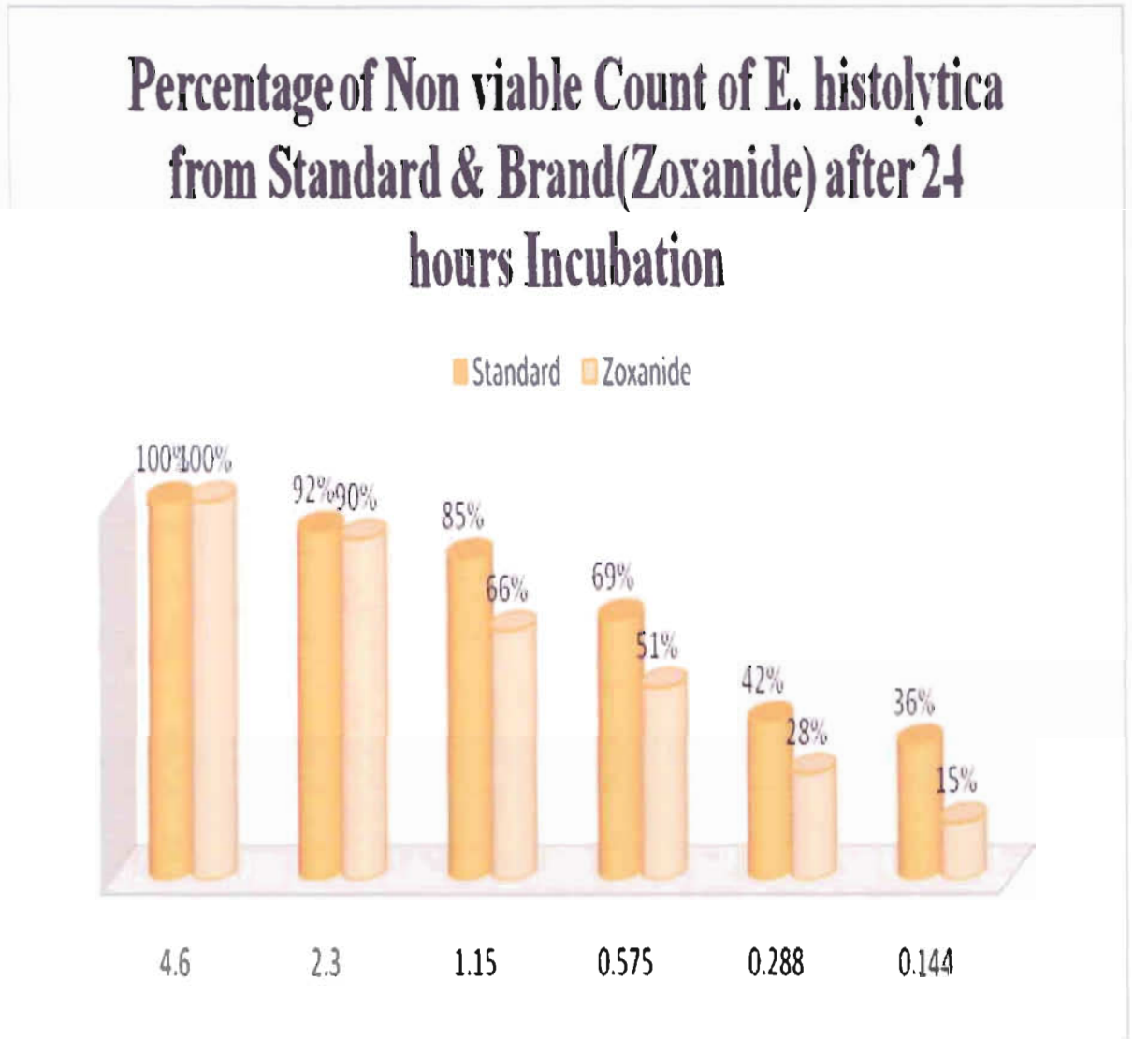
- After 24 hours incubation the non viable count of *Entamoeba histolytica* were 0.30×10^6 mL^{-1} and $0{\cdot}16\times10^6$ $mL^{-1}$when the concentration of Nitazoxanide was 0.144 µM for standard & brand respectively.

- In case of standard the non viable count of *E. histolytica* were 0.48×10^6 mL^{-1}, 0.78×10^6 mL^{-1} , 0.97×10^6 $mL^{-1}$and 1.05×10^6 mL^{-1} when the concentration were 0.29, 0.58, 1.15 and 2.3 µM respectively. At the same time the non viable count from brand (Zoxanide) of *Entamoeba histolytica* were $0.32\times10^6mL^{-1}$, 0.58×10^6 mL^{-1} , 0.75×10^6 $mL^{-1}$and 1.02×10^6 $mL^{-1}$when the concentration were 0.287, 0.575, 1.15 and 2.3 µM respectively.
- The non viable count of *Entamoeba histolytica* was increased to 1.14×10^6 mL^{-1} and 1.14×10^6 mL^{-1} when the concentration of Nitazoxanide was increased to 4.6µM for standard and brand(Zoxanide) respectively.

Table 8: Percentage of non viable count

Concentration of Nitazoxanide (µM) Standard	Percentage of the non viable count	Concentration of Nitazoxanide (µM) Drug(Zoxanide)	Percentage of the non viable count
4.6	100%	4.6	100%
2.3	92%	2.3	90%
1.15	85%	1.15	66%
0.575	69%	0.575	51%
0.288	42%	0.288	28%
0.144	36%	0.144	15%

Graph 4: Percentage of non viable count of *E. histolytica* fron standard & brand after 24 hours Incubation.



4.4 Trial 3

4.4.1 Viable count of *E.histolytica* from standard & commercial product of Nitazoxanide (Zoxanide) after 24 hours incubation.

The initial counts of the parasite were **1.14 $\times 10^6$ mL^{-1}**. After 24 hours the parasite counts of the control were **1.31$\times 10^6$ mL^{-1}**. The number of parasites are increased in the control after 24 hours incubation.

Table 9. Viable count of *E. histolytica* from Standard & Brand (Zoxanide) after 24 hours incubation.

Concentration of Nitazoxanide (µM) Standard	Viable count of *E. histolytica*	Concentration of Nitazoxanide (µM) Drug(Zoxanide)	Viable count *E. histolytica*
4.6	0.00×10^{6} mL^{-1}	4.6	0.00×10^{6} mL^{-1}
2.3	0.06×10^{6} mL^{-1}	2.3	0.20×10^{6} mL^{-1}
1.15	0.12×10^{6} mL^{-1}	1.15	0.16×10^{6} mL^{-1}
0.58	0.34×10^{6} mL^{-1}	0.58	0.41×10^{6} mL^{-1}
0.29	1.12×10^{6} mL^{-1}	0.29	0.73×10^{6} mL^{-1}
0.14	1.01×10^{6} mL^{-1}	0.14	1.12×10^{6} mL^{-1}

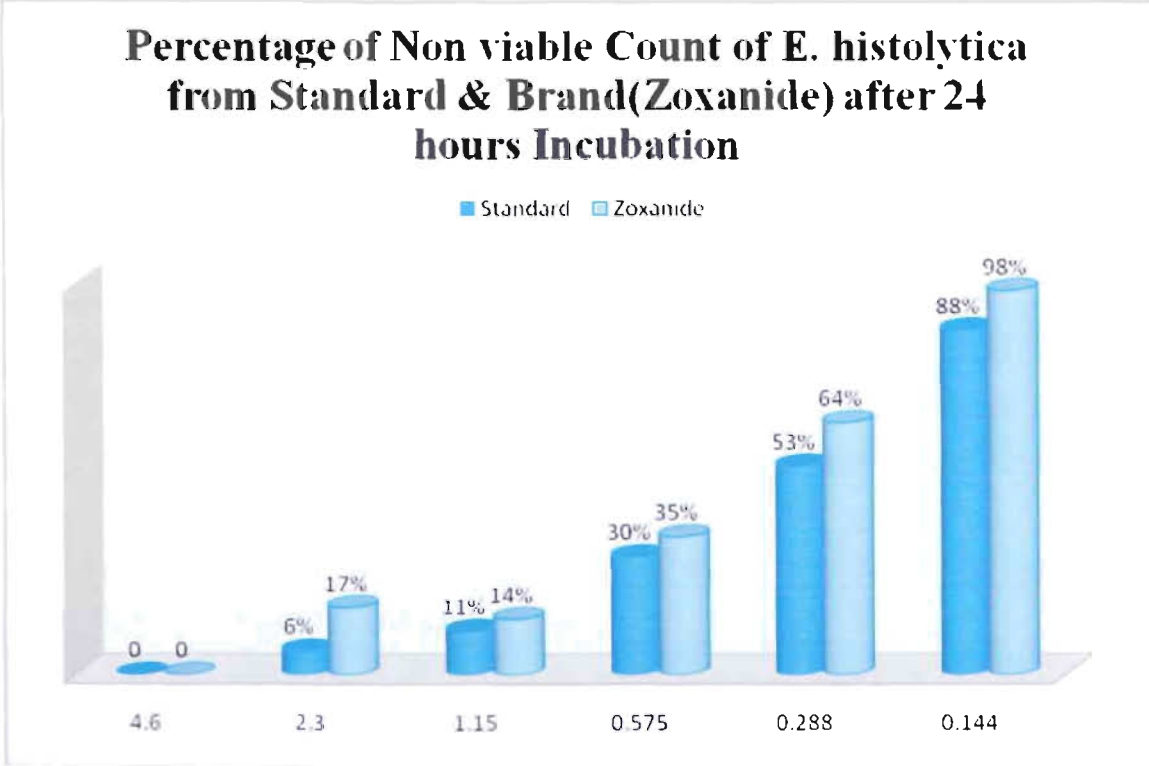
- After 24 hours incubation the viable count of *Entamoeba histolytica* were 1.01×10^{6} mL^{-1}& 1.12×10^{6} mL^{-1} for standard & brand respectively when the concentration of Nitazoxanide was 0·144 µM.
- The viable count of *Entamoeba histolytica* were 1.12×10^{6} mL^{-1}, 0.34×10^{6} mL^{-1}, 0.12×10^{6} mL^{-1} and 0.06×10^{6} mL^{-1}when the concentration were 0.29, 0.58, 1.15 and 2.3 µM respectively, when treated with the standard preparation. At the same time the viable count from brand (Zoxanide) of *Entamoeba histolytica* were 0.73×10^{6} mL^{-1}, 0.41×10^{6} mL^{-1}, 0.16×10^{6} mL^{-1} and 0.20×10^{6} mL^{-1}when the concentration were 0.287, 0.575, 1.15 and 2.3 µM respectively, when treated with the Zoxanide.

- The viable count of *Entamoeba histolytica* was decreased to 0.00×10^{6} mL^{-1} & 0.00×10^{6} mL^{-1} when the concentration of Nitazoxanide was increased to 4.6µM for standard and brand(Zoxanide) respectively.

Table 10: Percentage of the viable count

Concentration of Nitazoxanide (µM) Standard	Percentage of viable count	Concentration of Nitazoxanide (µM) Drug(Zoxanide)	Percentage of viable count
4.6	0	4.6	0
2.3	6%	2.3	17%
1.15	11%	1.15	14%
0.575	30%	0.575	35%
0.288	53%	0.288	64%
0.144	88%	0.144	98%

Graph 5. Percentage of viable count of *E. histolytica* from standard & commercial product (Zoxanide) of Nitazoxanide after 24 hours incubation.



4.4.2 Non viable count of *E. histolytica* from Standard & commercial product (Zoxanide) after 24 hours incubation.

Table 11. Non viable count of *E. histolytica* from Standard & Brand (Zoxanide) after 24 hours incubation.

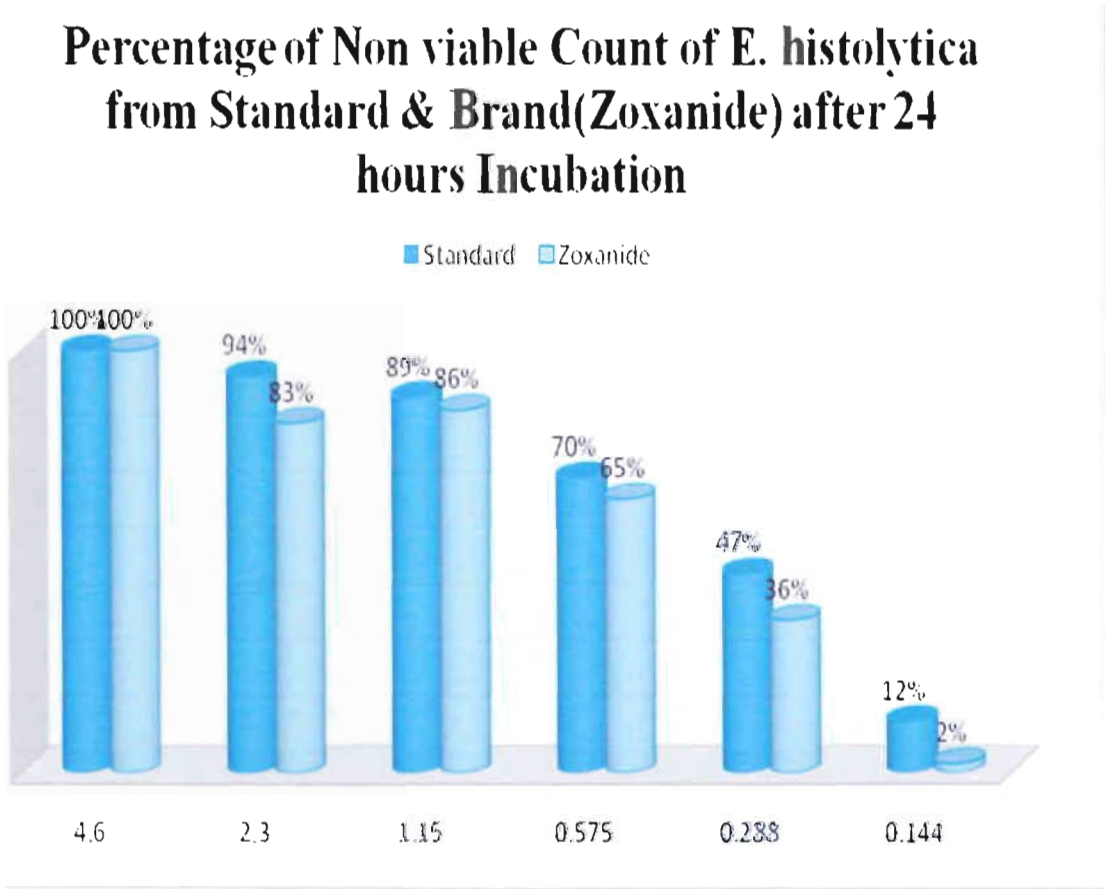
Concentration of Nitazoxanide (µM) Standard	Non Viable count of *E. histolytica*	Concentration of Nitazoxanide (µM) Drug(Zoxanide)	Non Viable count of *E. histolytica*
4.6	$1.14\times10^{6}\ mL^{-1}$	4.6	$1.14\times10^{6}\ mL^{-1}$
2.3	$1.07\times10^{6}\ mL^{-1}$	2.3	$0.94\times10^{6}\ mL^{-1}$
1.15	$1.02\times10^{6}\ mL^{-1}$	1.15	$0.98\times10^{6}\ mL^{-1}$
0.58	$0.80\times10^{6}\ mL^{-1}$	0.58	$0.73\times10^{6}\ mL^{-1}$
0.29	$0.53\times10^{6}\ mL^{-1}$	0.29	$0.41\times10^{6} mL^{-1}$
0.14	$0.13\times10^{6}\ mL^{-1}$	0.14	$0.02\times10^{6}\ mL^{-1}$

- After 24 hours incubation the non viable count of *Entamoeba histolytica* were $0.13\times10^{6}\ mL^{-1}$ and $0.02\times10^{6}\ mL^{-1}$ when the concentration of Nitazoxanide was 0.144 µM for standard & brand respectively.
- In case of standard the non viable count of *E. histolytica* were $0.53\times10^{6}\ mL^{-1}$, $0.80\times10^{6}\ mL^{-1}$, $1.02\times10^{6}\ mL^{-1}$ and $1.07\times10^{6}\ mL^{-1}$ when the concentration were 0.29, 0.58, 1.15 and 2.3 µM respectively. At the same time the non viable count from brand (Zoxanide) of *Entamoeba histolytica* were $0.41\times10^{6} mL^{-1}$, $0.73\times10^{6}\ mL^{-1}$, $0.98\times10^{6}\ mL^{-1}$ and $0.94\times10^{6}\ mL^{-1}$ when the concentration were 0.287, 0.575, 1.15 and 2.3 µM respectively.
- The non viable count of *Entamoeba histolytica* was increased to $1.14\times10^{6}\ mL^{-1}$ and $1.14\times10^{6}\ mL^{-1}$ when the concentration of Nitazoxanide was increased to 4.6µM for standard and brand(Zoxanide) respectively.

Table 12: Percentage of the non viable count

Concentration of Nitazoxanide (µM) Standard	Percentage of the non viable count	Concentration of Nitazoxanide (µM) Drug(Zoxanide)	Percentage of the non viable count
4.6	100%	4.6	100%
2.3	94%	2.3	83%
1.15	89%	1.15	86%
0.575	70%	0.575	65%
0.288	47%	0.288	36%
0.144	12%	0.144	2%

Graph 5. Percentage of non viable count of *E. histolytica* from standard & commercial product (Zoxanide) of Nitazoxanide after 24 hours incubation.



CHAPTER 5:
Discussion

5. Discussion:

Amoebiasis caused by *E. histolytica* is one of the most common parasitic infections of mankind. Research on different aspects of the parasite has been carried out in various parts of the world (Fotedar R. et, al. 2007). With regard to the global prevalence of the infection, 480 million people harbouring *E. histolytica* worldwide with approximately 40 million developing overt clinical disease and 50-1,00,000 deaths yearly (Gatti S. et. al, 2002). It is effective in treating invasive intestinal amoebiasis and in eliminating *E. histolytica* colonization of the intestinal tract (Rossignol JF. et, al. 2007).

The objective of the present study was to determine and compare the therapeutic efficacy of standard Nitazoxanide and the brand Zoxanide. Therefore, in this study *E. histolytica* was clinically isolated. The cinically isolated *E. histolytica* were treated with different concentration of brand Zoxanide and standard nitazoxanide. The experimental concentration were 0.07, 0.14, 0.28, 0.58, 1.15, 2.3, 4.6 μM. A control was made without the brand Zoxanide and the standard Nitazoxanide. The preparation was incubated for 24 hours. Finally the viable and non viable counts of *Entamoeba histolytica* were counted and recorded in which demonstrated that Nitazoxanide having good sensitivity against clinical isolates of *Entamoeba histolytica.*

In this study a total number of three trials were carried out. The initial count was 1.14×10^6 mL^{-1}. The brand Zoxanide was compared with the standard nitazoxanide at different concentrations. It was found that both the brand Zoxanide and standard nitazoxanide was showed about 100% inhibition of *E. histolytica* at the concentration of 4.6 μM. By observing all the data generated from the three trials, it has been found that upon increasing the concentration of Zoxanide the percentage of the viable count is decreased.

It is particularly difficult to compare the results of different studies because different methods are used for activity measurement. Some researchers use minimal lethal concentration (MLC), some MIC and some IC_{50} (Adagu S. et, al. 2002).

It has been stated that, Nitazoxanide has a documented broad spectrum of activity against parasites and anaerobic bacteria (Andrew H. et, al. 2006). Nitazoxanide was the most potent against *E. histolytica*, based on both the IC_{50} and IC_{90} values. The

susceptibility assays described herein showed that in the case of *E. histolytica*, both the IC_{50} and the IC_{90} were lower for nitazoxanide than for metronidazole. The IC_{50} value of nitazoxanide was 0.017 at the 95% limit. Concentrations as low as 17 ng/ml of nitazoxanide were able to halve the growth of *E. histolytica* trophozoites and 0.776 μg/ml was sufficient to inhibit growth by 90%, indicating that nitazoxanide possesses potent amebicidal activity (Roberto C. et, al. 2002). In this present study it has been found that the inhibition of the growth of *E. histolytica* was around 90% at the concentration of 2.3 μM.

It can be concluded that the *in vitro* sensitivity study of Zoxanide against clinically isolated *E. histolytica* is effective when compared to the standard at the concentration of 2.3 μM and above.

CHAPTER 6:
CONCLUSION

6. Conclusion:

On a global basis, amebiasis affects approximately 50 million persons each year, resulting in nearly 100,000 deaths. *Entamoeba histolytica* can also spread through food or water contaminated with stools. This contamination is common when human waste is used as fertilizer. It can also be spread from person to person- particularly by contact with the mouth or rectal area of an infected person

Nitazoxanide, a nitrothiazole benzamide, is an antiprotozoal agent. It has activity against *Cryptosporidium parvum* and *Giardia lamblia. In vitro* and or *in vivo* activity has also been seen with *Entamoeha histolytica, Trichomonas vaginalis, Enterocytozoon bieneusi, Giardia intestinalis, Clostridium difficile* and Helicobacterpy on. Nitazoxanide to be an inhibitor of pyruvate:ferredoxin oxidoreductase (PFOR) of *H. pylori.*

In this experimental the concentration of Nitazoxanide (Zoxanide) were 0.07, 0.14, 0.28, 0.58, 1.15, 2.3, 4.6 μM to a cliniclally isolated *Entamoeha histolytica.* A control group was made to measure the change in the viable counts and was put into the ELISA plate. Then the percentage of the viable and non viable amoeba was counted.

After the study it was conformed that Nitazoxanide and commercially available brand Zoxanide are effective in the treatment of a broad range of parasitic infections. The *in vitro* sensitivity of Nitazoxanide against *Entamoeba histolytica* is high and it is an innovative treatment option against amoebiasis.

CHAPTER 7:
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7. References

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