# *In-vitro* sensitivity study of Nitazoxanide against clinical isolates of *Entamoeba histolytica.*

A thesis report submitted to the Department of Pharmacy, East West University, Bangladesh, in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy.

Department of Pharmacy

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# CERTIFICATE

This is to certify that the thesis "*In vitro* sensitivity study of nitazoxanide 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 **Mymuna Mohsin Rahman** (ID: 2006-2-70-024) under my guidance and supervision and that no part of the thesis has been submitted for any other degree. I further certify that all the sources of information and laboratory facilities availed of this connection is duly acknowledged.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ELISA | Enzyme-linked immunosorbent assay |
| IgG | Serum antiiectin antibodies |
| IFA | Immunofluorescent assay |
| IA | Indirect hemagglutination |
| CIE | Counter-immunoelectrophoresis |
| CF | Complement fixation |
| PCR | Polymerase chain reaction |
| MHC | Major-histocompatibility-complex |
| MIF | Merthiolate-iodine-formaldehyde |
| PFOR | Pyruvate ferredoxin oxidoreductase enzyme |
| PBS | Phosphate buffered saline |
| MS-F | Modified Shaffer-Frye medium |
| WHO | The World Health Organization |
| Gal/GalNAc | specific V-acetyl-D-galactosamine lectio |
| ICDDR'B | International Centre for Diarrheal Disease Research, Bangladesh |

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# ABSTRACT

**Background:** *Entamoeba histolytica* is the etiological agent of amoebic dysentery and amoebic liver abscess. Amoebiasis caused by *Entamoeba histolytica* associated with high morbidity and mortality continues to be a major public health problem throughout the world. Asymptomatic individuals account for almost 90% of the infections. Diarrheal disease is the major cause of morbidity and mortality in children in developing countries. Poverty, ignorance, overcrowding, poor sanitation and malnutrition favor transmission and increased disease burden.

**Objectives:** To identify, describe the symptoms of *E.histolytica* and analysis of the interacting facts and cure of *E.histolytica* or more precisely in vitro sensitivity study of different brands of Nitazoxanide against clinical isolates of *E.histolytica.*

**Materials and methods:** The parasite count was adjusted to $6.3\times10^5$ $mL^{-1}$ in a medium. *In vitro* drug sensitivity assay of the Nitazoxanide was carried out by using microtiter plates after treatment with different concentration of Nitazoxanide. The experimental concentrations were 0.07, 0.14, 0.28, 0.58, 1.15, 2.3 and 4.6 μM. The viable parasites were counted by haemocytometer.

**Result:** After 24 h incubation the percentages of non viable count *of Entamoeba histolytica* was 83.34% and the viable count was 18.65% when the concentration of Nitazoxanide was 0.07 μM. After 24 h incubation the percentages of non viable count was 94.84% and the viable count was 5.15%when the concentration of Nitazoxanide was 4.6 μM and the initial count was $6.3\times 10^5$ $mL^{-1}$

**Conclusion:** The result shows that, Nitazoxanide is effective in the treatment of a broad range of parasitic infections. So, the In vitro sensitivity of Nitazoxanide against *Entamoeba histolytica* is high and it is an innovative treatment option against amoebiasis.

# CHAPTER ONE

# INTRODUCTION

## .1 Introduction

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*, *Entamoeba dispar,* and *Entamoeba moshkovskii*. Each year, more than 50 million individuals are infected, resulting in more than 100,000 deaths worldwide. Generally, infection caused by nonpathogens *E. dispar* and *E. moshkovskii* are approximately 10 times more frequent than infection caused by invasive *E. histolytica*. The indistinguishable morphology of *E. histolytica*, *E. dispar*, and *E. moshkovskii* makes it important to diagnose amebiasis using an *E. histolytica* specific test such as the Technology Lab antigen detection test or polymerase chain reaction (PCR) assay.

Amebiasis is a worldwide infection and is commonly found in tropical and subtropical countries. It is endemic to poor areas in developing countries. The majority of the morbidity and mortality is in Central and South Americas, Africa, and the Indian subcontinent. *E. histolytica* is found only in human hosts and amebiasis is commonly transmitted by a fecal–oral route through food and water contamination with fecal matter. Transmission also occurs by sexual practice (through oral-anal contact).

The life cycle of *E. histolytica* is simple. The enteric protozoan parasite can exist as either a cyst or a motile, vegetative trophozoite form. The cyst is responsible for the transmission of infection. It is a metabolically reduced tetra-nucleated cell and resistant to desiccation as well as other environmental factors. The infection is attributable to contamination of food or drinking water by the cyst form. Once a cyst is ingested by a new host, it lives in the ileum and transforms to the trophozoite form of the organism yielding four and then eight trophozoites. The trophozoite can colonize colonic epithelial cells of the bowel lumen and invades into the intestinal epithelium of the host. Adherence of *E. histolytica* trophozoites to host surfaces colonic mucin, epithelium, and other target cells, as well as a variety of cell lines is mediated by the amebic galactose (Gal) and N-acetyl d-galactosamine (GalNAc), a specific lectin. After adherence the parasite kills the epithelial cells, causing dysentery with blood and mucus in the stool. Immunologic and antigenic differences are distinguishable between *E. histolytica* and the nonpathogenic *Entamoeba* with the use of monoclonal antibodies; significant systemic humoral immune responses occur only with *E. histolytica* not with *E. dispar* or *E. moshkovskii* infection.

*E. histolytica* also secretes proteases that degrade the extracellular matrix and permit invasion of the bowel wall. Furthermore, *E. histolytica* can spread through the portal circulation and cause amebic liver abscesses. The infection may spread further by direct extension from the liver or through the bloodstream to the lungs, brain, and other organs.

The Gal/GalNAc lectin is a logical vaccine candidate because of its critical role in pathogencity. Genetic conservation of the Gal/GalNAc lectin between isolates may reveal that the lectin is under well-built, well-designed assortment or that *E. histolytica* is a clonal population. The high

sequence conservation of the lectin heavy subunit reveals that immune responses against it could be broadly cross protective.

## 1.2 Clinical Manifestations

Basically, the clinical classifications of the disease caused by *E. histolytica* are asymptomatic colonization and symptomatic disease (intestinal and extra-intestinal). The wide spectrum of the clinical intestinal disease ranges from asymptomatic carrier state to a fulminate colitis with an array of manifestations that may include toxic mega colon, perianal ulcer, peritonitis, and cutaneous amebiasis.

Most infections are in the form of asymptomatic (up to 90%) colonization, which is commonly caused by the nonpathogenic protozoa *E. dispar* and *E. moshkovskii* ; *E. histolytica* also frequently causes asymptomatic infection. In the United States and Europe, most isolates from homosexual men are *E. dispar*. In the case of asymptomatic colonization with *E. histolytica*, all subjects should be treated at least by luminal agents. If these individuals are not treated, they may be hazardous environmentally or may develop amebic colitis within months. Intestinal amebiasis can masquerade or simply be confused with bacillary dysentery, intestinal schistosomiasis, ulcerative colitis, acute fulminate dysentery, inflammatory bowel disease, ischemic colitis, diverticulitis and carcinoma of the colon.

## 1.3 Symptoms of amebic colitis/dysentery

- Abdominal pain or tenderness
- Diarrhea (watery/bloody/mucous)
- Tenesmus
- Flatulence
- Decrease of appetite
- Loss of weight
- Dehydration

Fever is uncommon. In the colon, inflammation and lesions may display as thickening of the mucosal wall, flask-shaped ulcerations or necrosis of the intestinal wall depending on the grade of invasion. Amebic liver abscess (ALA) is the most common manifestation of extra-intestinal amebiasis. The clinical presentation of ALA commonly includes fever, cough, abdominal pain or tenderness in right upper quadrant (in acute stage), enlarged liver, weight loss, fever, and abdominal pain (in subacute stage). Although patients with ALA may report a history of dysentery within the last year, in most patients it is impossible to identify the organism in stool.

ALA is a disease predominantly of young men, whereas pyogenic liver abscess (PLA) is commonly seen in older population of patients between ages 50 and 70 years, in equal ratios of

... to women. Both ALA and PLA are usually located in the right lobe of the liver, and both ALA and PLA are multiple in approximately 50% of all cases. Patients with PLA have a tendency to present with symptoms ranging in a period anywhere from 2 weeks to 1 or more months. Other clinical manifestations more common in patients with PLA include concurrent diabetes mellitus (DM) in approximately 27% of patients and biliary disease, jaundice, pruritus, elevated bilirubin and alkaline phosphates levels, sepsis, and positive bacterial cultures of blood or abscess fluid.

## 1.4 Parasites

- *Dientamoeba fragilis,* which causes *Dientamoebiasis*
- *Entamoeba dispar*
- *Entamoeba hartmanni*
- *Entamoeba coli*
- *Entamoeba moshkovskii*
- *Endolimax nana and*
- *Iodamoeba butschlii.*

Except for *Dientamoeba,* the parasites above are not thought to cause disease.

Amebiasis is a disease caused by the parasite *Entamoeba histolytica.* It can affect anyone, although it is more common in people who live in tropical areas with poor sanitary conditions. Diagnosis can be difficult because other parasites can look very similar to *E. histolytica* when seen under a microscope. Infected people do not always become sick. If your doctor determines that you are infected and need treatment, medication is available.

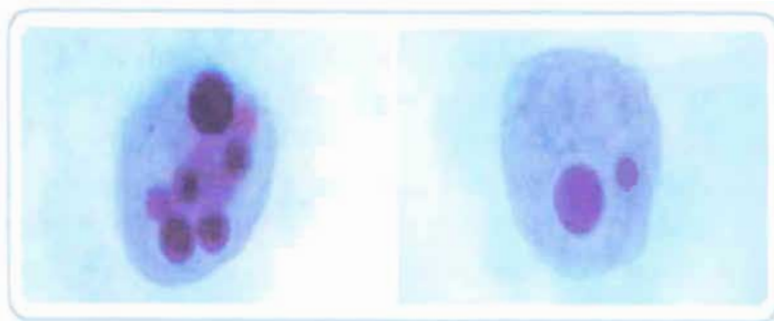


Figure: 1.1 Trophozoites of *E. histolytica* with ingested erythrocytes (red blood cells) stained with trichrome.

## 1.5 Free living Amebiasis

Amebiasis belonging to the genera *Naegleria, Acanthamoeba* and *Balamuthia* are free-living, amphizoic and opportunistic protozoa that are ubiquitous in nature. These amebas are found in soil, water and air samples from all over the world. Human infection due to these amebas involving brain, skin, lung and eyes has increased significantly during the last 10 years. The epidemiology, immunology, protozoology, pathology, and clinical features of the infections produced by these protozoa differ strikingly.

*Naegleria fowleri* (also known as "the brain-eating amoeba") is a free-living infection by the pathogenic, it is acquired by exposure to polluted water in ponds, swimming pools and man-made lakes. Raised temperatures during the hot summer months or warm water from power plants facilitate the growth of *N. fowleri. N. fowleri* is a thermophilic ameba that grows well in tropical and subtropical climates. The CNS infection, called Primary Amebic Meningoencephalitis (PAM), produced by *N. fowleri* is characterized by an acute fulminate meningoencephalitis leading to death 3–7 days after exposure. Victims are healthy, young individuals with a history of recent water-related sport activities. The portal of entry is the olfactory neuroepithelium. The pathologic changes are an acute hemorrhagic necrotizing meningoencephalitis with modest purulent exudates, mainly at the base of the brain, brainstem and cerebellum. Trophozoites can be seen within the CNS lesions located mainly around blood vessels. Thus far 179 cases have been reported; 81 in the USA alone.
*Balamuthia mandrillaris* and several species of *Acanthamoeba* are pathogenic "opportunistic" free-living amebas which cause Granulomatous Amebic Encephalitis (GAE) in humans and animals. GAE is an infection, usually seen in debilitated, malnourished individuals, in patients undergoing immunosuppressive therapy for organ transplants and in Acquired Immuno deficiency Syndrome (AIDS). The granulomatous component is negligible, particularly in immune compromised individuals. Pathologically these amebas produce a patchy, chronic or subacute granulomatous encephalitis with the presence of trophozoites and cysts. The portal of entry is probably through the respiratory tract or an ulceration of the skin reaching the CNS by hematogenous spread. As of October 1, 1996, 166 cases 1103 due to *Acanthamoeba* and 63 due to Balamothial of GAE have been reported from around the world. Of these 103 cases due to *Acanthamoeba* (72 have been reported in the USA alone, > 50 in AIDS). It is well known that several species of *Acanthamoeba* can also produce chronic sight threatening ulceration of the cornea called *Acanthamoeba* keratitis (AK), mostly in contact lens wearers or in individuals with minor corneal abrasions. Hundreds of cases of AK have been documented worldwide.

## 1.6 Diagnosis

There are several methods used for amebiasis diagnosis and monitoring of *E. histolytica* infection. Microscopy is a relatively nonspecific and insensitive but widely available diagnostic test for *E. histolytica, E. dispar*, and *E. moshkovskii*. Enzyme linked immuno assays (ELISAs)

detect *E. histolytica* antigen in the stool, where available, have replaced the traditional microscopy for diagnosis of *E. histolytica*.

Serologic tests are useful for diagnosis of infection. The traditional but time consuming immunofluorescence, counter immuno electrophoresis, indirect hemagglutination assays (IHAs) have been supplanted in most laboratories by tests based on enzyme-linked immuno sorbent assay or *E. histolytica* specific antigen (ELISA). However, serologic techniques are not entirely satisfactory because of inadequate sensitivity and specificity in the detection of early infections and the fact that serologic assays remain positive for months to years after exposure to the parasite.

Several newer molecular diagnostic tests that facilitate accurate monitoring of amebiasis have become available. The ELISA/DNA detection based test (PCR) and culture remain the principal means of diagnosis of infection in individuals. Rapid diagnosis and the early treatment of clinical cases are important in reducing the morbidity and mortality in amebiasis. Several PCR-based assays for the detection of the *E. histolytica* infecting humans have been described, and the results of evaluating these molecular tests have been encouraging, indicating sensitivities and specificities higher than those achievable by routine microscopy. Newer molecular diagnostic tests provide important insights into the state and level of activity of *E. histolytica* infection and will continue to be used more widely, especially among patients with severe infection or in case of require to interpretation in immuno compromised patients. Now the antigen detection ELISAs offer the capability of identifying and distinguishing *E. histolytica* from *E. dispar* and *E. moshkovskii* infections.

## .7 Current Diagnosis

### Intestinal Amebiasis

A clinical presentation such as diarrhea/dysentery (remember many infections are asymptomatic).
Cconventional microscopic examination is insensitive and cannot distinguish invasive *E. histolytica* from the nonpathogenic *Entamoeba dispar*.
Test the stool sample with the *E. histolytica* specific EIA or real time PCR, and also perform a serology (*E. histolytica* antibody detection based test on serum sample EIA, IFA, IHA, LA, etc.).

### Amebic Liver Abscess (ALA)

Obtain travel history to endemic areas/residence immigrant from developing country for amebiasis, age (20–40 years), history of alcohol abuse, and gender (male/female 9:1).
History of having had dysentery within the last year.
Cclinical manifestations (i.e., abdominal pain localized in right upper quadrant, fever, point

nderness over the liver, hepatomegaly).
Obtain biochemical laboratory findings (i.e., leukocytosis, mild anemia, elevated nsaminase/alkaline phosphates levels).
Screen for presence of abscess with imaging analyses and obtain the *E. histolytica* specific A or real time PCR testing on stool sample, and/or serology (*E. histolytica* antibody detection sed test on serum sample EIA, IFA, IHA, LA, etc.).
Differentiate pyogenic liver abscess with some findings (i.e., age older than 40, women more n men, concurrent diabetes mellitus, and biliary disease) and positive cultivation for bacteria the blood and abscess fluid.

veral studies indicate that early detection of amebiasis based on genomic amplification (as is ne with PCR) may provide an adjunct and may be a more sensitive method than conventional ethods. Because of the development of molecular biology–based diagnostic tests such as the CR, it is possible to detect low numbers of cyst and trophozoites of *E. histolytica* in clinical ecimens; these tests also enormously powerful tools for genetically typing different amoebic rains. Particularly, real-time PCR is a rapid and sensitive method.

## 8 Treatment

here are a number of drugs available that are effective in treating amebiasis. The major drugs ed to treat asymptomatic cyst passers are Tinidazole(tindamax), Iodoquinol (Yodoxin), and iloxanide furoate (Furamide), **Secnidazole (Sindose),** a no absorbable amino glycoside, has the vantage of being poorly absorbed, making it possible to use in pregnancy, and well tolerated in ildren. Iodoquinol (Yodoxin) is a highly effective luminal agent but has some gastrointestinal de effects and may interfere with thyroid function tests caused by high iodine content. iloxanide furoate (Furamide) is relatively nontoxic and is used as a luminal agent worldwide vailable only from the Centers for Disease Control and Prevention in the United States).

## .9 Current Therapy

**ntestinal Amebiasis**

Consider medical therapy for asymptomatic cyst passers: paromomycin or iodoquinol or loxanide furoate (available in the United States only from the CDC).
Remember paromomycin may be useful during pregnancy.
For mild to moderate intestinal amebiasis (diarrhea/dysentery), consider metronidazole or nidazole or ornidazole followed by paromomycin.
For severe intestinal amebiasis, metronidazole or tinidazole followed by paromomycin is commended.

**Amebic Liver Abscess (ALA)**

Metronidazole or tinidazole followed by paromomycin is recommended.

The drug of choice for the therapy of invasive intestinal amebiasis and amebic liver abscess is metronidazole (Flagyl) or the related (and now available in the United States) drug tinidazole (Tindamax). Metronidazole (Flagyl), a nitroimidazole, is given in divided doses for 7 days, with an efficacy of more than 90%. It has a high gastrointestinal absorption and is tolerated well in infants and children. Metronidazole (Flagyl) may also be associated with a disulfiram like reaction to alcohol when consumed with the drug. The most effective available therapeutic regimen is the combination of metronidazole followed by (not given concurrently) a luminal agent such as paromomycin (or iodoquinol) in intestinal amebiasis. Because the teratogenic effect of metronidazole seems greatest during the first trimester, it can possibly be used safely in the last two trimesters of pregnancy in severe amebiasis. Tinidazole, another nitroimidazole, has shown tremendous efficacy when given in doses of 2mg orally once daily for 3 days; as mentioned above, this drug is now available in the United States. Response to drug therapy depends on the cyst size, location, and host immunity and also is the best monitored by serial imaging and serology. Indications for therapeutic draining of ALA are:

- Clinically a lack of response to drug therapy over 3 to 7 days. Normally patients with ALA regularly respond up to 5 days to metronidazole therapy with a decline in fever and right-upper quadrant pain.
- High possibility of ruptured abscess (large) as characterized by cavity size larger than 5 cm
- A left-lobe abscess that appears to be ruptured into pericardium.
- A differential diagnosis still including pyogenic abscess.

## 1.10 Relative Frequency of the Disease

In older textbooks it is often stated that 10% of the world's population is infected with *Entamoeba histolytica*. It is now known that at least 90% of these infections are due to *E. dispar*. Nevertheless, this means that there are up to 50 million true *E. histolytica* infections and approximately seventy thousand die each year, mostly from liver abscesses or other complications. Although usually considered a tropical parasite, the first case reported (in 1875) was actually in St Petersburg in Russia, near the Arctic Circle. Infection is more common in warmer areas, but this is both because of poorer hygiene and the parasitic cysts surviving longer in warm moist conditions.

## 1.11 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

## 1.12 Nature of the disease

Most infected people, perhaps 90% are asymptomatic, but this disease has the potential to make the sufferer dangerously ill. It is estimated by the World Health Organization that about 70,000 people die due to amoebiasis annually worldwide.

Infections can sometimes last for years. Symptoms take from a few days to a few weeks to develop and manifest themselves, but usually it is about two to four weeks. Symptoms can range from mild diarrhea to dysentery with blood and mucus. The blood comes from amoebae invading the lining of the intestine. In about 10% of invasive cases the amoebae enter the bloodstream and may travel to other organs in the body. Most commonly this means the liver, as this is where blood from the intestine reaches first, but they can end up almost anywhere.

Onset time is highly variable and the average asymptomatic infection persists for over a year. It is raised that the absence of symptoms or their intensity may vary with such factors as strain of amoeba, immune response of the host, and perhaps associated bacteria and viruses.

In asymptomatic infections the amoeba lives by eating and digesting bacteria and food particles in the gut, a part of the tract. It does not usually come in contact with the intestine itself due to

the protective layer of mucus that lines the gut. Disease occurs when amoeba comes in contact with the cells lining the intestine. It then secretes the same substances it uses to digest bacteria, which include enzymes that destroy cell membranes and proteins. This process can lead to penetration and digestion of human tissues, resulting first in flask shaped ulcers in the intestine.

*Entamoeba histolytica* ingests the destroyed cells by phagocytosis and is often seen with red blood cells inside when viewed in stool samples. Especially in Latin America, a granulomatous mass (known as an amoeboma) may form in the wall of the ascending colon or rectum due to long-lasting immunological cellular response, and is sometimes confused with cancer Theoretically, the ingestion of one viable cyst can cause an infection.

## 1.13 *Entamoeba histolytica*

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

*Entamoeba histofytica* is the etiologieal agent of amoebic dysentery and amoebic liver abscess (ALA). Worldwide, 40-50 million symptomatic cases of amoebiasis occur annually affii 70,000 to 100.000 deaths due to this infection. There are two distinct, but morphologically identical species of Entamoeba: *Entamoeba histofytica,* is pathogenic (Walsh, 1986)

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 (Ghosh, 1998).

*Entamoeba* associated infection is a public health problem in many developing countries, where it is responsible for severe morbidity and mortality (Ghosh, 2000).

Worldwide there is the higher incidence of amebiasis in developing countries. Diarrheal diseases continue to be major causes of morbidity and mortality in children in developing countries. For example, in Bangladesh 1 in 30 children dies of diarrhea or dysentery by her fifth birthday (Haque, 2003)

## 1.14 History of *Entamoeba histolytica*

Human infections of the parasite are not a recent phenomenon. The earliest record of symptoms of the disease bloody, mucose diarrhea was from the Sanskrit document *Brigu-samhita*, written at around 1000BC. Assyrian and Babylonian texts also have references to the diseases, with descriptions of blood in the feces, thus suggesting that amoebiasis occurred in the Tigris-Euphrates basin before the sixth century BC. Later records were able to distinguish bacterial infections with those of amoebic origin: epidemics of dysentery by itself are more likely to result from bacterial infections, while dysentery that is associated with disease of the liver is more likely to be caused by amoeba. Thus, around the second century AD, there was clearer understanding of the association between liver abscesses and amoebas. Around the 16th century, amoebiasis became more widespread in the developed world, mostly due to the growth of European colonies and increased world trade. There had been many clear descriptions of the hepatic and intestinal forms of amoebiasis, considered as the cause of a "bloody flux" spreading through Europe, Asia, Persia, and Greece. The first accurate description of both forms of the disease came from the book Researches into the Causes, Nature and Treatment of the More Prevalent Diseases of India and of Warm Climates Generally by James Annersley, written in the 19th century.

Considering their small size, protozoa were difficult to identify before the invention of the microscope in the 17th century. The causal agent, *Entamoeba histolytica*, was discovered in Russia in 1873 by Friedrich Losch .His early observations came from the case of a young farmer who had been suffering from chronic dysentery. In his diagnosis, Losch found large numbers of of amoeba in his feces and associated the amoebas to be the cause of the dysentery

For a long time, it was known that people who were infected with *E. histolytica* never developed symptoms and spontaneously clear the infection, i.e. those who were shedding *E. histolytica* cysts in their stools did not show symptoms of the disease. In 1925, Emile Brumpt suggested that there must be two species: one that is invasive while the other is not (which he named *E. dispar*). But like so many new ideas in science, his hypothesis was dismissed by others. In 1969, WHO defined amoebiasis as "infection with *Entamoeba histolytica*, with or without clinical manifestations", thus implying that all the strains were potentially pathogenic, yet never answering the question of way some people ended up to be asymptomatic. It was not until the 1970s that supporting data for Brumpt's hypothesis accumulated. In 1993, due to biochemical, immunological and genetic data that supports this view, a formal re-description of *E. histolytica* was published with the invasive species named *E. histolytica* and the non-invasive species named *E. dispar*. A 1997 WHO meeting in Mexico City led to clear guidelines distinguishing the two species.

## 1.15 Transmission

The active (trophozoite) stage exists only in the host and in fresh loose feces; cysts survive outside the host in water, soils and on foods, especially under moist conditions on the latter. The cysts are readily killed by heat and by freezing temperatures, and survive for only a few months outside of the host. When cysts are swallowed they cause infections by excysting (releasing the trophozoite stage) in the digestive tract. The pathogenic nature of *E. histolytica* was first reported by Losch in 1875, but it was not given its Latin name until Fritz Schaudinn described it in 1903. *E. histolytica,* as its name suggests (*histo–lytic* = tissue destroying), is pathogenic; infection can lead to amoebic dysentery or amoebic liver abscess. Symptoms can include fulminating dysentery, bloody diarrhea, weight loss, fatigue, abdominal pain, and amoeboma. The amoeba can actually 'bore' into the intestinal wall, causing lesions and intestinal symptoms, and it may reach the blood stream. From there, it can reach different vital organs of the human body, usually the liver, but sometimes the lungs, brain, spleen, etc. A common outcome of this invasion of tissues is a liver abscess, which can be fatal if untreated. Ingested red blood cells are sometimes seen in the amoeba cell cytoplasm.

## 1.16 Scientific classification of *Entamoeba histolytica*

Common name: Ameba

Phylum: Protozoa

Class: Archamoebae

Order: Amoebida

Family: Entamoebidae

Genus: Entamoeba

## 1.17 Characteristics of *Entamoeba histolytica*

| Genus and Species | *Entamoeba histolytica* |
|---|---|
| Biologic 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);<br><br>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 |

## 1.18 Life Cycle of *Entamoeba histolytica*

The uninucleate trophozoite of *Entamoeba histolytica* inhabits the colon and rectum and at times, the lower end of the small intestine of the humans and other primates (figure: 1.2). The motile trophozoite measures an average 25 $\mu$ m in diameter (range 15 to 60 $\mu$ m) and is typically monopodial, producing one large, finger-like pseudopodium at a time (Figure: 1.1). The single pseudopodium erupts and is withdrawn so rapidly that, in prepared slides, trophozoites with pseudopodia extended are rarely seen. The cytoplasm is differentiated into two zones: a clear, refractile ectoplasm and a finely granular endoplasm in which food vacuoles occur. Such vacuoles may contain host erythrocytes, leukocytes, and epithelial cells, as well as bacteria and other intestinal material. Trophozoites proliferate mitotically (binary fission) within the host's gut (Burton, 2005).

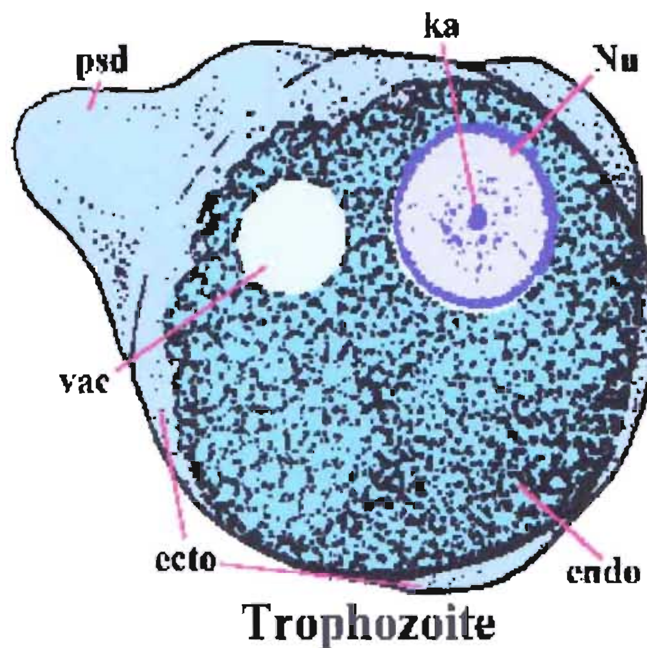



Figure: 1.2: Entamoeba histolytica Trophozite Early cysts with chromatoidal bars, later cyst (Burton 2005)

The nucleus is of special importance in differentiating *E. histolytical* from most of the other intestinal amoebae. In saline preparations, the nucleus has a barely discernible nuclear envelope. However, in stained preparations, the vesicular nucleus is clearly visible. Ideally, it has a well-defined envelope, lined on the inner surface with fine peripheral chromatin granules and a minute, centrally located endosome. Unfortunately, this ideal morphology is not confined to *E.*

*histolytica.* Often, other species of *Entamoeba,* notably *E. dispar*, show similar nuclear morphologies (Burton, 2005).

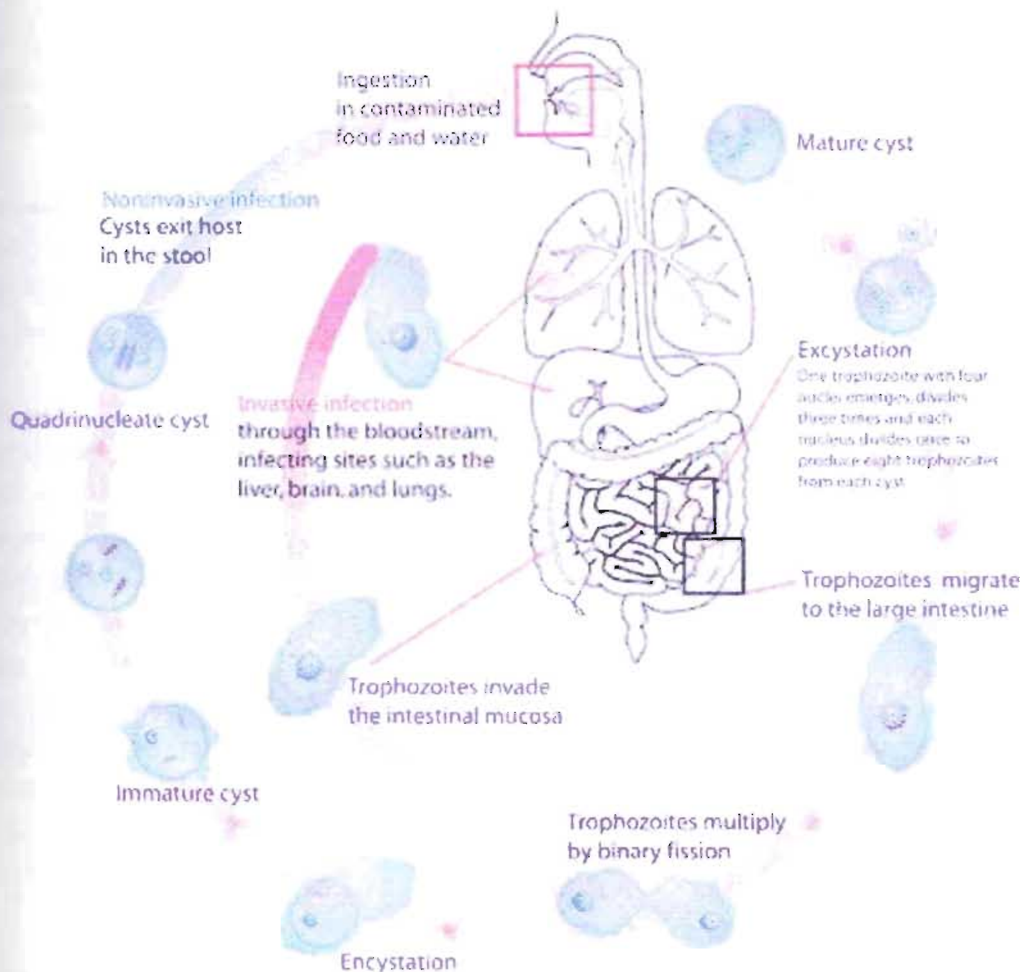



Figure-1.3: Life cycle of *Entamoeba histolytica* (Burton, 2005)

Under certain adverse environment and/or physiological circumstances, throphozoites assume precystic characteristics by becoming more spherical and as food vacuoles are extruded, shrinking in size. Pseudopodia, if formed, are sluggishly extended and there appears to be no progressive movement. Encystations' begins with the secretion by the precyst trophozoite of a thin, surrounding hyaline membrane to form a cyst wall. At this stage, the cyst is usually spherical, an average 12μm in diameter (range 10 to 20μm) with a single nucleus. At times, glycogen masses and chromatoidal bars may be observed (Figure: 1.2). (Burton, 2005).

Cysts of *E. histolytica* are highly resistant to desiccation and even to certain chemicals. Cysts in water can survive for a month, while those in feces on dry hand can survive for more than 12 days; they Tolerate temperatures up to a thermal death : point of 50°C (Burton, 2005).

When food or water contaminated with *E. histolytica* cysts is ingested by a host, the eysts pass through the stomach to the ileum, where encystation occurs. The neutral or slightly alkaline environment afforded by the small intestine is apparently requisite for this phenomenon. However, *in vitro* studies suggest that excystation does not occur immediately; cysts placed in fresh culture (Burton, 2005).

Second only to the liver in frequency as an extra intestinal sit are the lung. Pulmonary Amoebiasis is relatively rare, however and when seen is probably a direct result of hepatic infection. Unlikely most amoebic abscesses which are commonly bacteriological sterile, the pulmonary abscess is often vulnerable to secondary bacterial infections (Burton, 2005).

Immunity to infection with *E. histolytica* is associated with a mucosal IgA response against the carbohydrate recognition domain of the Gal/GalNA clectin. Over a one-year period, children with this response had 86% fewer new infections than children without this response. Cell-mediated responses have been described in patients with amebic liver abscess, characterized by lymphocyte proliferation and lymphokine secretion that is amebicidal in vitro. One study found that in patients with liver abscess, the prevalence of the class II MHC haplotype FfLA-DR3 is increased by a factor of more than three, suggesting a role of CD4+ f cell function in the outcome of the disease. It is not worth however that the acquired syndrome pandemic has not led to increases in invasive amebiasis although asymptomatic intestinal colonization is undoubtedly common. In fact, in the murine model of amebic colitis, the depletion of CD4+ T cells decreases the severity of the disease (Haque, 2003).

## 1.19 Pathogenesis

The major limitation one faces in studying pathogenesis is the lack of a satisfactory animal model which can duplicate the spectrum of human disease. Nonetheless several species have been used as animal models to study various aspects of pathogenesis (Meerovitch and Chadee, 1988). For example, hamsters and gerbils are most commonly used as models for liver disease. Trophozoites produce lesions when injected directly into the liver of these animals. *In vitro* models are also available for studying various steps involved in pathogenesis (Petri and Ravdin, 1988). For example, adherence can be scored by using Chinese hamster ovary (CHO) cells, erythrocytes or bacteria. Lysis can be scored as per cent cell culture monolayers disrupted. The number of erythrocytes ingested per trophozoite can be used as a measure of phagocytosis. One or more experimental approaches have been taken to study the killing of target cells by

*[his]tolytica* trophozoites. 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 (Bracha *et al* 1982). Virulence of trophozoites of strain 200: NIH varied depending on culture associates. When cultured with NR[S] 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 (Ravdin and Guerrant 1981). 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 (Ravdin *et al* 1985b). Two surface molecules responsible for adherence have been identified one inhabitable by galactose or N-acetyl-D-galactosamine (GalNAc) (Bracha and Mirelman 1983; Petri *et al* 1987; Ravdin and

Guerrant 1981; Ravdin *et al* 1985c) and the other inhibitable by N-acetyl-D-glucosamine (GlcNAc) polymers (Kobiler and Mirelman 1981). 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 (reviewed in McCoy 1994). 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 (Chadee 1987, 1988; Ravdin and Guerrant 1981; Ravdin *et al* 1985a; Salata *et al* 1985a; Salata and Ravdin 1986), (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 (Mann *et al* 1991; Purdy *et al* 1993; Tannich *et al* 1991). 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) (Mann *et al* 1991; Purdy *et al* 1993; Tannich *et al* 1991). Two light subunit genes (*hgl* 1-2) have also been identified and characterized (McCoy *et al* 1993a, b; Tannich *et al* 1992). 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 (McCoy *et al* 1993b). 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 (Pollard 1976); the optimal temperature being 37°C. Studies with

the $Ca^{2+}$-binding fluorescent dye FURA-2 showed 20-fold increase in intracellular $Ca^{2+}$ in target cells within seconds of direct contact. Actual cell death occurred 5-15 min after the lethal hit. Possible roles of $Ca^{2+}$ are in contact dependent release of cytotoxic enzymes and toxins, cytoskeletal changes and activation of $Ca^{2+}$-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 (Bos *et al* 1980). It is believed that these thiol-proteases may be one of the molecules involved in pathogenesis (McKerrow 1993). This is based on the fact that there seems to be a correlation between clinical severity 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 (Gadasi and Kobiler 1983; Lushbaugh *et al* 1989). Patients with invasive disease produce antibodies against this enzyme; those with non-invasive disease do not (Reed *et al* 1989). The enzyme has broad substrate specificity. It can utilize casein, gelatin, insulin, type I collagen, fibronectin and laminin as substrates (Keene *et al* 1986; Luaces and Barrett 1988; Scholze and Schulte 1988; Scholze and Werries 1986; Schulte *et al* 1987). It is a cathepsin B-like enzyme. Similar enzymes are found in extracellular milieu of invasive tumour cells (Lushbaugh 1988). 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 (Leippe *et al* 1991, 1992). 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 $\alpha$ 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 (Tsutsumi *et al* 1984). 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 (Guerrant *et al* 1981). 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 1985b).

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 (McCaul 1977). 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.

## 1.20 Hostess Defense

### 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. A newly developed gnotobiotic model of intestinal amebiasis should enable testing of this hypothesis and provide clues to help design practical studies in humans.

### *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.

# CHAPTER TWO

# TREATMENT OF *E. HISTOLYTICA*

## 2.1 Treatment of *E. histolytica:*

*E. histolytica* infections occur in both the intestine and (in people with symptoms) in tissue of the intestine and/or liver. As a result two different sorts of drugs are needed to rid the body of the infection, one for each location. Metronidazole, or a related drug such a tinidazole, is used to destroy amoebae that have invaded tissue. It is rapidly absorbed into the bloodstream and transported to the site of infection. Because it is rapidly absorbed there is almost none remaining in the intestine. Since most of the amoebae remain in the intestine when tissue invasion occurs, it is important to get rid of those also or the patient will be at risk of developing another case of invasive disease. Several drugs are available for treating intestinal infections, the most effective of which has been shown to be Secnidazole; diloxanide furoate; ornidazole; Diloxmd is used in the US. Both types of drug must be used to treat infections, with metronidazole usually being given first, followed by Diloxmd.

## 2.2 Treatment of amebiasis

### A. 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
- Fulminant colitis requiring surgical evaluation
- Peritonitis and suspected amebic liver abscess rupture

### B. Surgical Care

- Prompt surgical evaluation is needed in suspected cases of fulminate colitis, peritonitis, or perforated viscous.
- Surgical intervention is usually indicated in different clinical scenarios: uncertain diagnosis (possibility of pyogenic liver abscess); concern of bacterial super infection in amebic liver abscess; failure to respond to metronidazole after 4-day treatment duration; emphysema after amebic liver abscess rupture; large left-sided amebic liver abscess representing 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 improves the outcome in the treatment of amebic emphysema and is life-saving in amebic pericarditis.

- 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 (>300 $cm^3$).

## C. Consultations

- Infectious disease specialist
- General surgeon
- Gastrointestinal specialist

## D. Medication

The following drugs and medications are in some way related to, or used in the treatment of Amebiasis. This service should be used as a supplement to, and not a substitute for, the expertise, skill, knowledge and judgment of healthcare practitioners.

### 2.2.1 Secnidazole:

Secnidazole is a nitroimidazole anti-infective drug which is effective in the treatment of dientamoebiasis .

**Used in the Treatment**:

- Intestinal ameobiasis
- Fiardiasis
- Trichomoniasis
- Bacterial vaginosis

It is said that such disease symptoms can be treated with a single once only dose of Secnidazole.

**Side Effects:**

The drug has certain side effects that can affect individuals in different ways. The following are some of the side effects that are often associated with the drug:

- Loss of appetite
- Nausea
- Stomach Discomfort
- Diarrhea
- Skin Rash
- Fatigue
- Headache

### 2.2.2 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

### 2.2.3 Tinidazole: Tinidazole an anti-parasitic drug used against protozoan infections.

**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
- Headache

**2.2.4 Diloxanide Furoate:** Diloxanide Furoate is an anti-protozoal drug used in the treatment of *Entamoeba histolytica* and some other protozoal infections.

**Used in the Treatment:**

- Long-term gut infection causing diarrhea (chronic amoebic dysentery)
- Sudden severe diarrhea due to infection caused by the amoeba *Entamoeba histolytica* (acute amoebic dysentry).

**Side Effects:**

- Itchy rash (urticaria)
- Itching (pruritus)
- Excess gas in the stomach and intestines (flatulence).

**2.2.5 Metronidazole:** Metonidazole is a nitroimidazole antibiotic medication used particularly for anaerobic bacteria and protozoa.

**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

# CHAPTER THREE

# NITAZOXANIDE

## 3.1 Nitazoxanide

Nitazoxanide, also known by the brand names Alinia and Annita (and by Daxon, Dexidex, Kidonax, Pacovanton and Paramix in Mexico, by Nitax, 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.

## 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. Nitazoxanide was successful in the treatment of metronidazole-resistant giardiasis. Studies have suggested efficacy in the treatment of cyclosporiasis, isosporiasis, and amebiasis.

## 3.2 Mechanism of action

The anti-protozoal activity of nitazoxanide is believed to be due to interference with the pyruvate: ferredoxin oxidoreductase (PFOR) enzyme dependent electron transfer reaction which is essential to anaerobic energy metabolism.

It has also been shown to have activity against influenza A virus.The mechanism appears to be by selectively blocking the maturation of the viral hemagglutinin at a stage preceding resistance to endoglycosidase H digestion. This impairs hemagglutinin intracellular trafficking and insertion of the protein into the host plasma membrane.

## 3.3 Uses

Nitazoxanide is a first line choice for the treatment of illness caused by *Cryptosporidium parvum* or *Giardia lamblia* infection in immuno competent 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 *Cryptospridium 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, in combination with peginterferon alfa-2a and ribavirin.

A randomised double-blind placebo-controlled study published in 2006, with a group of 38 young children (Lancet, vol 368, page 124-129) concluded that a 3-day course of nitazoxanide significantly reduced the duration of rotavirus disease in hospitalized pediatric patients. Dose given was "7.5 mg/kg twice daily" and the time of resolution was "31 hours for those given nitazoxanide compared with 75 hours for those in the placebo group." It is to be noted that rotavirus is the most common infectious agent associated with diarrhea in the pediatric age group worldwide.

## 3.4 Pharmacokinetics:

Nitazoxanide is a prodrug.

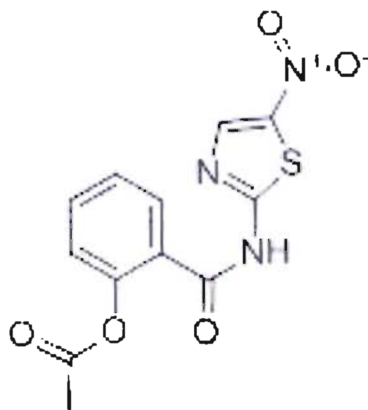
Following oral administration, it is rapidly hydrolyzed to its active metabolite, tizoxanide which is 99% protein bound. Peak concentrations are observed 1–4 hours after administration.It is excreted in the urine, bile and feces.

## 3.5 Dosage forms:

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

## 3.6 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 |
| Weight: | 307.28 |

## Nitazoxanide Chemical Properties:

MP: 202 C

## Adverse reaction of Nitazoxanid:

- Diarrhea;
- Headache;
- Nausea;
- Stomach pain.

**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;
- Unusual tiredness.

## 3.8 Warning & 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 have 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.

## 3.9Drug 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).

## 3.10 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 FOUR

# AIM & SIGNIFICANCE

# Aim of study

Amebiasis is a common disease caused by the protozoan *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. As a fact Bangladesh is among third world countries and a big percentage of its population cannot afford expensive medication.

There are a lot of researches have taken place to develop a promising cure of amebiasis and prominently Nitazoxanide is one of them. 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.

Our aim of this research paper is to identify, describe the symptoms of *E.histolytica* and analysis of the interacting facts and cure of *E.histolytica* or more precisely in vitro sensitivity study of different brands of Nitazoxanide against clinical isolates of *E.histolytica*.

## Significance of study

A developing country like Bangladesh always faces problem in medication. Poor medication and sanitation system prolongs its inability of securing total cure against even some common diseases like, Amebiasis. Amebiasis mainly caused by the protozoan *E. histolytica*. It 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 Amebiasis completely. This research paper significantly discus about the main causes of Amebiasis, types of Amebiasis, symptoms, diagnosis, treatment and medication. Research paper also significantly promotes in vitro sensitivity study of different brands of Nitazoxanide against clinical isolates of *E.histolytica.* Nitazoxanide is one of the powerful drugs against Amebiasis and it is significantly effective against Amebiasis. Along with the other facts of *E.histolytica* this research paper emphasizes on the history of Nitazoxanide, chemical details, mechanism of action, pharmacokinetic, doses, how to use and as well as the adverse reactions of the drug. It is obvious that this research paper may not provide all the information about Amebiasis and drug Nitazoxanide but surely it can help significantly for future research and development on Amebiasis and its' drugs.

# CHAPTER FIVE

# MATERIAL & METHODS

## Materials and Methods

### 5.1 Research design

The research design in the " In Vitro Sensitivity of Nitazoxanide againest clinical isolation of *Entamoeba histolytica"*

### 5.2 Clinical isolation

Clinical isolates from patients attending the Out Patient Departments of ICDDRB hospital, attached to the Parasitology Laboratory, ICDDRB, Dhaka, Bangladesh, identified earlier as either *Entamoeba histofytica* by Techlab ELISA were used in the present study. These have been cultured in Robinson's medium (Robinson GL, 1968).

### 5.3Preparation 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:

### Xenic Culture Media

**Preparation of Riee 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.

## Diphasic media

**Robieson's medium:** Robinson's medium is a complex medium that has nevertheless found widespread use for the isolation of enteric amoeba. Robinson's medium is prepared with the six following stock solutions.

*(a) 0.5% **erythromycin*** 0.5% erythromycin is prepared in distilled water and filtered and sterilized. Then it is refrigerated.

***(b)** 20% **Bacto Peptone.*** 20% Bacto Peptone is prepared in distilled water. Then it is autoclaved and refrigerated.

***(c)* 10*X* phthalate solution *stock.*** 10 X phthalate solution stocks are prepared by mixing 102 g of potassium hydrogen phthalate and 50 ml of 40% sodium hydroxide. The solution is made to 1 liter at pH is adjusted to 6.3. Then the solution is autoclaved for 15 min at 121 °C under a pressure of 15 Ib/in2. The solution is stored at room temperature. It is diluted 1:10 with sterile water before use. A stock of phthalate-Bacto Peptone can be made by adding 1.25 ml of 20% Bacto Peptone per 100 ml of 1 X phthalate solution. The solution is refrigerated.

***(d) 10X R medium stock.*** 10X R medium stock, is prepared by dissolving the following in-distilled water: 25.0 g of sodium chloride; 10.0 g of citric acid; 25.0 g of potassium phosphate, monobasic; 5.0 g of ammonium sulfate; 0.25 g of magnesium sulfate. 7H2O; and 20 ml of 85% lactic acid solution. It is made to 500 ml.Stock is diluted to 1:10, pH is adjusted to 7.0. The solution is autoclaved for 15min at 121°C under a pressure of 15 lb/in$^2$ in 20-ml amounts.

***(e) BR medium.*** To prepare BR medium, inoculate 1 x R medium with a standard *E. coli* strain.Incubated at 37°C for 48 hours and store at room temperature (good for several months).

***(f) BRS medium.*** To prepare BRS medium, added an equal volume of heat-inactivated bovine serum to BR medium and incubate at 37°C for 24 hours. Store at room temperature (good for several months).

## 5.4 Prepareation of agar slants

To prepare agar slants, many people use 1/2 oz.Quorak bottles (total volume, 7 ml), but we have also used standard culture tubes with good success. Autoclave a solution of 1.5% Noble agar in 0.7% sodium chloride-distilled water for 15 min at 121°C under a pressure of 15 Ibs. Dispense in 5-ml (tube) or 7-ml (bottle) amounts, reautoclave, and slant until cool and set. For slants in tubes, use an angle that produces a 12- to 15-mm (ca. 0.5-in.) butt.

When cool, tighten lids and store at room temperature or refrigerated. To one tube or bottle add the following: 3 ml of 1 xphthalate-Bacto Peptone, 1 ml of BRS, 50μl erythromycin. This must be done on the same day as inoculation.

## 5.5 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.

*E. histafytica.* LYI-S-2: In the course of developing YI-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 (unpublished data).

## 5.6 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.

**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.

**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.

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.

**Isolations:** Our experience of LE medium 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 by others 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 of 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 mucous or bloody if these are present. Stool fractionation by flotation in zinc sulfate or sucrose 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 in which one has antibiotics added and the other does not. Penicillin streptomycin and erythromycin are the antibiotics of choke, as they appear to have little direct effect on the amoebea. 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 h before examination. A drop of sediment can be extracted from the tube for examination on a microscope slide. Alternatively, cultures can be examined in situ 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 and above the slant in diphasic media. In situ examination is much easier in monophasic medium due to its relative clarity. If no growth is observed at 48 h, a blind passage should be made.

Most of the liquid overlying the sediment is discarded to leave less than 1 ml in the tube. In sediment is resuspended in the remaining fluid and transferred to a fresh culture tube with medium and rice. After incubation for an additional 48 hours the culture is reexamined as described above. If no amoebae are seen further 48-hours incubation is warranted, and this is followed by reexamination. If there are still no amoebae seen, the culture is discarded as negative. If cultures are positive for amoebae, it is usually helpful to centrifuge the cultures in a swinging-bucket rotor and divide the pellet among the recipient tubes. This can be done by chilling the culture tubes for 5 min in an ice-water bath, inverting several times to detach adherent amoebae, and transferring the liquid phase to an empty culture tube before centrifugation. Cultures in LE medium can also be pelleted,

But in our experience the agar slant of Robinson's medium is not as conducive to this approach. As growth improves, centrifugation is no longer necessary as measured macula (<2 ml) can be transferred to the fresh medium.

## 5.7 Axenization

As previously mentioned, axenization of *E. histolytica* is a long and laborious procedure involving gradual adaptation of the parasite to a new way of life. A brief overview of the method is given here. The medium can be a specialized monoxenic culture medium as described previously, but we have also had success initiating such cultures using one of the axenic media such as TYI-S-33.

The monoxenic associate we have used most frequently is C. *fasciculate.* This insect flagellate is grown as a stock culture at room temperature and added to the monoxenic culture of amoebae at each subculture, as *Crithidia* does not grow at the incubation temperature of the amoebae; the amount added varies. *T. cnizi* Culbertson has also been used successfully as the associated organism but is not recommended due to the potential for infection, even though this strain is of very low virulence. The antibiotics added vary both in type and amount depending on the sensitivities of the flora in which the amoebae were growing. We have used a cocktail of rifampin, amikacin, oxytetracycline, and cefotaxime with good success. Except for the first agent, those are effective on the amoeae.

The initial concentration is often as high as 0.1 mg/ml of culture medium. After 24 h, the cells are pelleted by centrifugation and the medium is replaced. As the ameba cell numbers increase, the cell pellet can be divided between two tubes. By reducing the antibiotic concentration gradually in one of a pair of tubes to test for bacterial growth, sterility can be achieved gradually while at the same time the numbers of amoebae are increasing.

At least two subcultures in the absence of antibiotics should be performed before the cultures can be considered free of bacteria. This can be verified using standard aerobic and anaerobic testing procedures for bacteria, including mycoplasmas, and fungi. Established monoxenic cultures, those in which growth is reproducible and bacteria are absent, are then used to initiate axenic cultures.

This uses the same medium but with no *Crithidia* added. After a few subcultures the flagellates disappear as a result of dilution and ingestion. It is often helpful, although not always necessary, to add a small amount of Noble agar to the tubes.It appears to form a substrate for the amoebae. In addition, the tubes should be incubated vertically rather than at 5° to the horizontal, as this appears less "stressful" to the cells. Often the culture will flourish initially and then numbers will crash. It is at this crisis point that the cultures are most vulnerable. As long as a few live cells persist, it is worth continuing to replace the medium every few days. With luck, the numbers will gradually start to increase again, and eventually addition of the agar will no longer be needed. When established, the axenic cultures can be incubated at 5° to the horizontal and eventually should reach cell concentrations of 150,000 to 300,000 per ml on a twice-per-week subculture.

## 5.8 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.

## 5.9 Protocol of Encystment

(a) 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.

(b) Day 4.

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

(c) Day 6.

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

(d) 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 h.

e) 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.

## 5.10 Protocol: encystment 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.

## 5.11 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.

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-fourth.

## 5.12 Maintenance of cultures

Established cultures of all parasites are handled la essentially the same way. Xenk cultures of *E. histolytica* are routinely passaged at 48- to 72-h intervals; usually a

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)*.

## 5.13 Preparation of Antimicrobial agent

Nitazoxanide drug is a standard used in the study was collected as pure salt from ACI Pharmacuticals LTD,Dhaka,Bangladesh and aslo some other diffrent brand. standard Nitazoxanide was weighed and dissolved in 1mL of distilled water. In a refrigerator the stock solution was stored.

## In vitro drug sensitivity assay

Drug sensitivity assay of the sample was carried out by using microtiter plated. In row A 200 micro liter of the standard was given and then sample were given. In all other rows (B-H) the 100 micro liter medium was added and dilution of the gruges were perfomed down the plate then mixed properly. 100 micro liter of the meduim from the last row (H) was discarded to maintain the quality of the concentrationof the drugs. 0.07, 0.14, 0.29,0.58, 1.15, 2.3 and 4.6 μM. Further 100μL 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 hour under a miroscope to check for thr presence of amoeba. After 4 hours the plate was taken the incubator. Then the viable parasites were counted by haemocytomete under microscope in each of the row.

# CHAPTER SIX

# RESULTS

## 6.1 Measurement of amoebicidal activity:

According to the study, Nitazoxanide having good amoebicidal activity. The clinical isolates of *Entamoeba histolytica* were treated with Nitazoxanide at different concentration. The experimental concentrations 0.14, 0.28, 0.58, 1.15, 2.3, and 4.6 μM. A control group was made to measure the change in the viable counts and was put into the ELISA plate. Each ELISA plate now contained different concentration of Nitazoxanide and some amount of *Entamoeba histolytica* (100 micro liters). After that the preparation was incubated for a definite period of time (24-48) hours.

Finally the viable and non viable counts of *Entamoeba histolytica* were counted and recorded in a table and in a table which demonstrated that Nitazoxanide having good sensitivity against clinical isolates of *Entamoeba histolytica.*

### 6.1.1 Viable count of *Entamoeba histolytica* after 24 hours incubation:

When the preparation was completed then it was incubated in 24 hours with different concentration of Nitazoxanide.

The initial count of the parasite was $6.3 \times 10^5$ $mL^{-1}$and the initial count of the control media were $4.3\times10^5$ $mL^{-1}$. After 24 hours the viable count *of Entamoeba histolytica* were $11.75\times10^4$ mL-$^1$when the concentration of Nitazoxanide was 0.07 μM and the viable count of *Entamoeba histolytica* were $10..5\times10^4$ $mL^{-1}$, $12.75\times10^4$ $mL^{-1}$, $14.75\times10^4$ $mL^{-1}$, $13\times10^4$ $mL^{-1}$and $6.5\times10^4$ $mL^{-1}$ when the concentration were 0.14, 0.28, 0.57, 1.15 and 2.3 μM respectively. The viable count of *Entamoeba histolytica* was decreased to $3.25\times10^4$ $mL^{-1}$ when the concentration of Nitazoxanide was increased to 4.6 μM.

The numbers of parasites are increased in the control it indicates that the numbers of parasite are increased after 24 hours incubation.

So, the percentages of the viable count are increased when the concentrations of Nitazoxanide are decreased.

**Table 6.1** Viable counts and it's percentages of *Entamoeba histolytica* after 24h lucubration

| Concentration (µM) | Viable parasites count ($mL^{-1}$) | Percentages of viable count of *Entamoeba histolytica* |
|---|---|---|
| 4.6 | $3.25\times10^{4}$ $mL^{-1}$ | 5.15% |
| 2.3 | $6.5\times10^{4}$ $mL^{-1}$ | 10.31% |
| 1.15 | $13\times10^{4}$ $mL^{-1}$ | 20.63% |
| 0.57 | $14.75\times10^{4}$ $mL^{-1}$ | 23.41% |
| 0.28 | $12.75\times10^{4}$ $mL^{-1}$ | 20.23% |
| 0.14 | $10.5\times10^{4}$ $mL^{-1}$ | 16.66% |

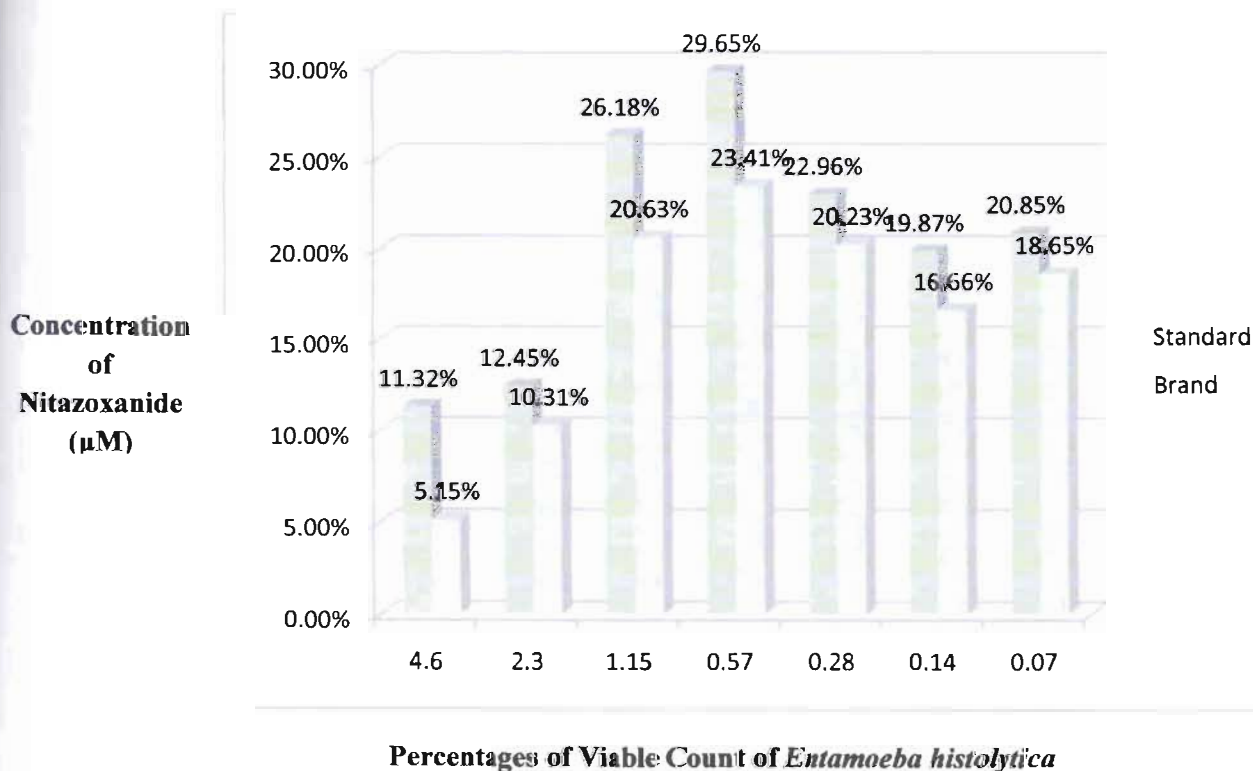



Figure 6.1 Percentages of Viable Count of the viable counts of *Entamoeba histolytica* after 24 hours incubation

### 6.1.2 Non Viable count of *Entamoeba histolytica* after 24 hours incubation:

When the preparation was completed then it was incubated in 24 hours with different concentration of Nitazoxanide.

**Table 6.2** Non Viable counts and it's percentages of *Entamoeba histolytica* after 24h lucubration

| Concentration (μM) | Non Viable parasites count ($mL^{-1}$) | Percentages of non viable count of *Entamoeba histolytica* |
|---|---|---|
| 4.6 | $5.97\times10^5$ $mL^{-1}$ | 94.84% |
| 2.3 | $5.65\times10^5$ $mL^{-1}$ | 89.68% |
| 1.15 | $5.0\times10^5$ $mL^{-1}$ | 79.36% |
| 0.57 | $4.82\times10^5$ $mL^{-1}$ | 76.58% |
| 0.28 | $5.02\times10^5$ $mL^{-1}$ | 79.76% |
| 0.14 | $5.25\times10^5$ $mL^{-1}$ | 83.33% |

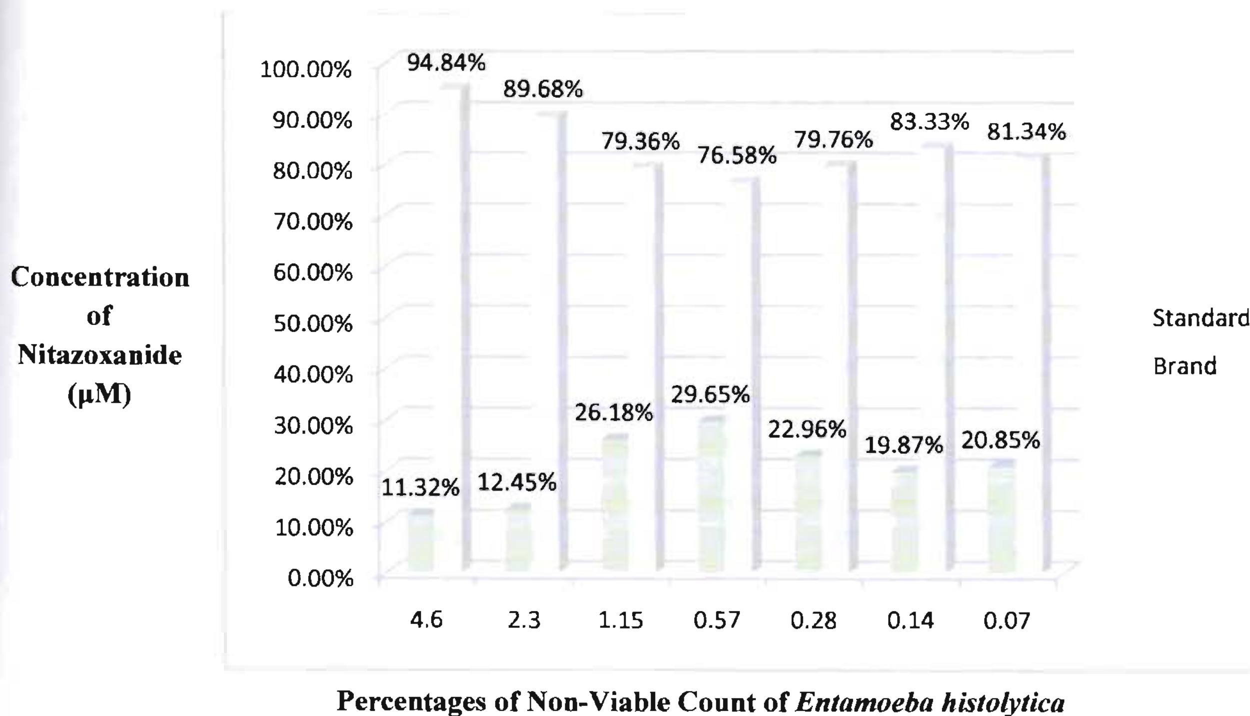



Figure 6.2 Percentages of the non viable Count of the viable counts of *Entamoeba histolytica* after 24 hours incubation

The initial count of the parasite was 6.3 $\times 10^5$ $mL^{-1}$ and the initial count of the control media were $4.3\times10^5$ $mL^{-1}$. After 24 hours the non viable count of *Entamoeba histolytica* was $5.12\times10^5$ $mL^{-1}$. When the concentration of Nitazoxanide was 0.07 μM and the non viable count of *E. histolytica* were $5.25\times10^5 mL^{-1}$, $5.02\times10^5$ $mL^{-1}$, $4.82\times10^5$ $mL^{-1}$, $5.0\times10^5$ $mL^{-1}$ and $5.65\times10^5$ $mL^{-1}$. When the concentration were 0.14, 0.28, 0.57, 1.15 and 2.3 μM respectively. The non viable count of *Entamoeba histolytica* was increased to $5.97\times10^5$ $mL^{-1}$ when the concentration of Nitazoxanide was increased to 4.6 μM . The numbers of parasites are increased in the control it indicates that the numbers of parasite are increased after 24 hours incubation. So, the percentages of the non viable count are increased when the concentrations of Nitazoxanide are increased.

### 6.1.3 Percentages of Viable counts Non Viable counts of *Entamoeba histolytica* :

Table 6.3 Percentages of Viable counts Non Viable counts of *Entamoeba histolytica*

| Concentration of Nitazoxanide (μM) | Percentage of Viable count of *Entamoeba histolytica* | Percentages of non viable count of *Entamoeba histolytica* |
|---|---|---|
| 4.6 | 5.15% | 94.84% |
| 2.3 | 10.31% | 89.68% |
| 1.15 | 20.63% | 79.36% |
| 0.57 | 23.41% | 76.58% |
| 0.28 | 20.23% | 79.76% |
| 0.14 | 16.66% | 83.33% |

From the result it can be concluded that when the concentration is 4.6 μM then viable count is 5.15% and the non viable count is 94.84% .On the other hand when the concentration is 0.07 μM the viable count is 18.65% and non viable count is 81.34%.So when the concentrations of Nitazoxanide are increased then the viable count of *Entamoeba histolytica* are decreased and ultimately the non viable count of *Entamoeba histolytica* increased when the concentrations of the drug are increased. It show that Nitazoxanide having antiamoebic activity.

### 6.1.4 Viable count of *Entamoeba histolytica* after 24 hours incubation:

**Table 6.3** Viable counts and it's percentages of *Entamoeba histolytica* after 24h lucubration when the initial count is $7.1 \times 10^5$ $mL^{-1}$

| Concentration (μM) | Viable parasites count ( $mL^{-1}$) | Percentages of viable count of *Entamoeba histolytica* |
|---|---|---|
| 4.6 | $4.5\times10^4$ $mL^{-1}$ | 6.33% |
| 2.3 | $4.75\times10^4$ $mL^{-1}$ | 6.69% |
| 1.15 | $6\times10^4$ $mL^{-1}$ | 8.45% |
| 0.57 | $6.75\times10^4$ $mL^{-1}$ | 9.50% |
| 0.28 | $7.75\times10^4$ $mL^{-1}$ | 10.91% |
| 0.14 | $9\times10^4$ $mL^{-1}$ | 12.67% |

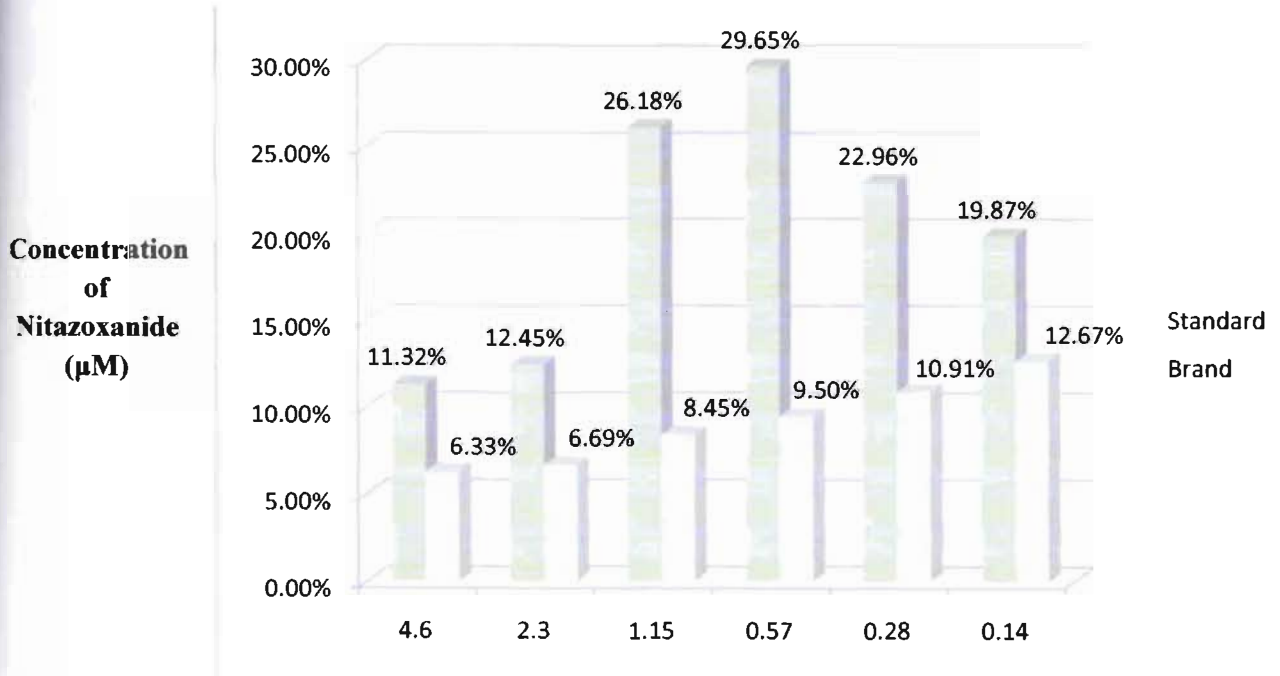



Figure 6.3 Percentages of Viable Counts of the viable counts of *Entamoeba histolytica* after 24 hours incubation when the initial count is $7.1 \times 10^5$ $mL^{-1}$

This figure shows that the viable count of *Entamoeba histolytica* after treating with different concentration of Nitazoxanide for 24 hours. The initial count of *Entamoeba histolytica* is 7.1 $\times 10^5$ $mL^{-1}$. After 24 h incubation the count of *Entamoeba histolytica* in the control media were 3.1 $\times 10^5$ $mL^{-1}$. After 24 hours the viable count were $9\times10^4$ $mL^{-1}$when the concentration of Nitazox was 0.14 μM and the other viable count of *Entamoeba histolytica* were $7.75\times10^4$ $mL^{-1}$, $6.75\times10^4$ $mL^{-1}$, $6\times10^4$ $mL^{-1}$, and $4.75\times10^4$ $mL^{-1}$ when the concentration were 0.28, 0.58, 1.15 and 2.3 μM respectively. When the concentration of Nitazoxanide was increased to 4.6 μM then the viable count were $4.5\times10^4$ $mL^{-1}$.So, the viable count are increased when the concentration of Nitazoxanide is decreased.

### 6.1.5 Non Viable count of *Entamoeba histolytica* after 24 hours incubation when initial count 7.1 $\times 10^5$ mL-$^1$.

When the preparation was completed then it was incubated in 24 hours with different concentration of Nitazoxanide. Then the flowing data was found.

**Table 6.4** Non Viable count of *Entamoeba histolytica* after 24 hours incubation when initial count 7.1 $\times 10^5$ $mL^{-1}$

| Concentration (μM) | Non Viable parasites count ($mL^{-1}$) | Percentages of non viable count of *Entamoeba histolytica* |
|---|---|---|
| 4.6 | $6.65\times10^5$ $mL^{-1}$ | 93.66% |
| 2.3 | $6.62\times10^5$ $mL^{-1}$ | 93.30% |
| 1.15 | $6.5\times10^5$ $mL^{-1}$ | 91.54% |
| 0.57 | $6.47\times10^5$ $mL^{-1}$ | 91.19% |
| 0.28 | $6.32\times10^5$ $mL^{-1}$ | 89.08% |
| 0.14 | $6.2\times10^5$ $mL^{-1}$ | 87.32% |

The initial count of the parasite was 7.1 $\times 10^5$ $mL^{-1}$.and the initial count of the control media were 3.1 $\times 10^5$ $mL^{-1}$. After 24 hours the non viable count of *Entamoeba histolytica* were $6.2\times10^5$ $mL^{-1}$ when the concentration of Nitazoxanide was 0.14 μM and the non viable count of *E. histolytica* were $6.32\times10^5$ $mL^{-1}$, $6.47\times10^5$ $mL^{-1}$, $6.5\times10^5$ mL-$^1$ , $6.62\times10^5$ $mL^{-1}$ and 6.65×105 $mL^{-1}$when the concentration were 0.14, 0.28, 0.57,1.15 and 2.3 μM respectively. The non viable count *of Entamoeba histolytica* were increased to 7.1 $\times 10^5$ $mL^{-1}$.When the concentration of Nitazoxanide was increased to 4.6 μM . The numbers of parasites are increased in the control it indicates that the numbers of parasite are increased after 24 hours incubation. So, the percentages of the non viable count are increased when the concentrations of Nitazoxanide are increased.

Figure 6.4 Percentages of non Viable Counts of the viable counts of *Entamoeba histolytica* after 24 hours incubation when the initial count is 7.1 $\times10^{5}$ $mL^{-1}$

### 6.1.6 Percentages of Viable counts Non Viable counts of *Entamoeba histolytica* when the initial count is 7.1 $\times10^{5}$ $mL^{-1}$

**Table 6.5** Percentages of Viable counts Non Viable counts of *Entamoeba histolytica* when the initial count is 7.1 $\times10^{5}$ $mL^{-1}$

| Concentration of Nitazoxanide (µM) | Percentage of Viable count of *Entamoeba histolytica* | Percentages of non viable count of *Entamoeba histolytica* |
|---|---|---|
| 4.6 | 6.33% | 93.66% |
| 2.3 | 6.69% | 93.30% |
| 1.15 | 8.45% | 91.54% |
| 0.57 | 9.50% | 91.19% |
| 0.28 | 10.91% | 89.08% |
| 0.14 | 12.67% | 87.32% |

From the result it can be concluded that when the concentration is 4.6 μM then viable count is 6.33% and the non viable count is 93.66%.On the other hand when the concentration is 0.14 μM the viable count is 12.67% and non viable count is 87.32% So when the concentrations of Nitazoxanide are increased then the viable count of *Entamoeba histolytica* are decreased and ultimately the non viable count of *Entamoeba histolytica* increased when the concentrations of the drug are increased. It show that Nitazoxanide having antiamoebic activity.

### 6.1.7 Viable count of *Entamoeba histolytica* after 24 hours incubation:

**Table 6.6** Viable counts and it's percentages of *Entamoeba histolytica* after 24h lucubration when the initial count is $6.5 \times 10^5$ $mL^{-1}$

| Concentration (μM) | Viable parasites count ($mL^{-1}$) | Percentages of viable count of *Entamoeba histolytica* |
|---|---|---|
| 4.6 | $4.75\times10^4$ $mL^{-1}$ | 7.30% |
| 2.3 | $5.5\times10^4$ $mL^{-1}$ | 8.46% |
| 1.15 | $5.75\times10^4$ $mL^{-1}$ | 8.84% |
| 0.57 | $7.25\times10^4$ $mL^{-1}$ | 11.15% |
| 0.28 | $8.25\times10^4$ $mL^{-1}$ | 12.69% |
| 0.14 | $10\times10^4$ $mL^{-1}$ | 15.38% |

When the preparation was completed then it was incubated in 24 hours with different concentration of Nitazoxanide. The initial count of the parasite were $6.5 \times 10^5$ $mL^{-1}$ and the initial count of the control media were $3.0 \times 10^5$ $mL^{-1}$ After 24 hours the viable count of *Entamoeba histolytica* was $10\times10^4$ $mL^{-1}$ when the concentration of Nitazoxanide 0.14 μM and the viable count of *Entamoeba histolytica* were $8.25\times10^4$ $mL^{-1}$, $7.25\times10^4$ $mL^{-1}$, $5.75\times10^4$ $mL^{-1}$and $5.5\times10^4$ $mL^{-1}$ when the concentration were 0.28, 0.57, 1.15 and 2.3 μM respectively. The viable count of Entamoeba histolytica was decreased to $4.75\times10^4$ $mL^{-1}$. When the concentration of Nitazoxanide was increased to 4.6 μM .

The numbers of parasites are increased in the control media it indicates that the numbers of parasite are increased after 24 hours incubation. So, the percentages of the viable counts are increased when the concentrations of Nitazoxanide are decreased.

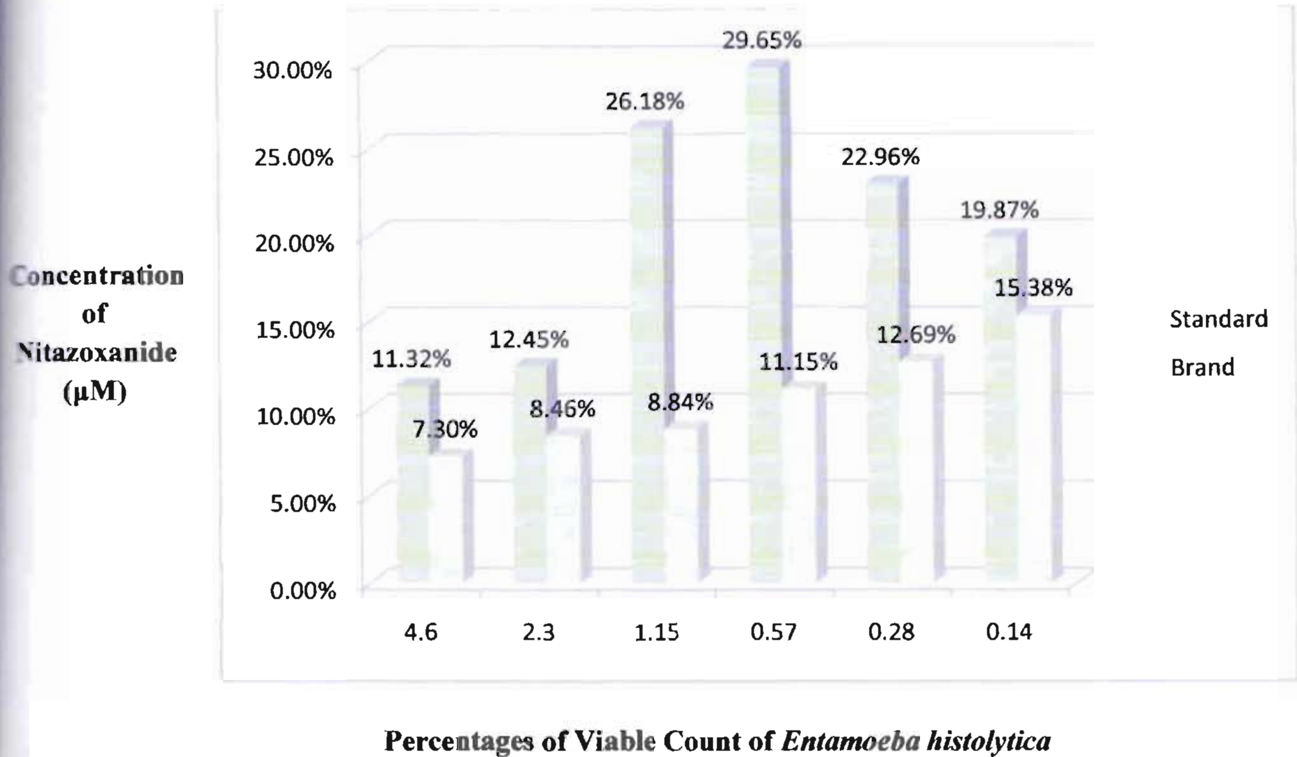



Figure 6.6 Percentages of Viable Counts of the viable counts of *Entamoeba histolytica* after 24 hours incubation when the initial count is $6.5 \times 10^5$ $mL^{-1}$

### 6.1.8 Non Viable count of *Entamoeba histolytica* after 24 hours incubation when initial count $6.5 \times 10^5$ $mL^{-1}$

When the preparation was completed then it was incubated in 24 hours with different concentration of Nitazoxanide. Then the flowing data was found.

**Table 6.7** Non Viable count of *Entamoeba histolytica* after 24 hours incubation when initial count $6.5 \times 10^5$ $mL^{-1}$.

| Concentration (µM) | Non Viable parasites count ($mL^{-1}$) | Percentages of non viable count of *Entamoeba histolytica* |
|---|---|---|
| 4.6 | $6.02\times10^5$ $mL^{-1}$ | 92.69% |
| 2.3 | $5.95\times10^5$ $mL^{-1}$ | 91.53% |
| 1.15 | $5.92\times10^5$ $mL^{-1}$ | 91.15% |
| 0.57 | $5.77\times10^5$ $mL^{-1}$ | 88.84% |
| 0.28 | $5.67\times10^5$ $mL^{-1}$ | 87.30% |
| 0.14 | $5.5\times10^5$ $mL^{-1}$ | 84.61% |

Figure 6.7 Percentages of Non Viable Counts *Entamoeba histolytica* after 24 hours incubation when the initial count is $6.5 \times 10^5$ $mL^{-1}$

The initial count of the parasites was $6.5 \times 10^5$ $mL^{-1}$ and the initial count of the control media were $3.0 \times 10^5$ $mL^{-1}$. After 24 hours the non viable count *of Entamoeba histolytica* were $5.5\times10^5$ $mL^{-1}$ when the concentration of Nitazoxanide was 0.14 μM and the non viable count of *E. histolytica* were $5.67\times10^5$ $mL^{-1}$ , $5.77\times10^5$ $mL^{-1}$ , $5.92\times10^5$ $mL^{-1}$ and $5.95\times10^5$ $mL^{-1}$ when the concentration were 0.28, 0.57, 1.15 and 2.3 μM respectively. The non viable count of *Entamoeba histolytica* was increased to $6.02\times10^5$ $mL^{-1}$ when the concentration of Nitazoxanide was increased to 4.6 μM . The numbers of parasites are increased in the control it indicates that the numbers of parasite are increased after 24 hours incubation. So. The percentages of the non viable count are increased when the concentration of Nitazoxanide are increased.

### 6.1.9 Percentages of Viable counts Non Viable counts of *Entamoeba histolytica* when the initial count is $6.5 \times 10^5$ $mL^{-1}$

**Table 6.7** Percentages of Viable counts Non Viable counts of *Entamoeba histolytica* when the initial count is $6.5 \times 10^5$ $mL^{-1}$

| Concentration of Nitazoxanide (μM) | Percentage of Viable count of *Entamoeba histolytica* | Percentages of non viable count of *Entamoeba histolytica* |
|---|---|---|
| 4.6 | 7.30% | 92.69% |
| 2.3 | 8.46% | 91.53% |
| 1.15 | 8.84% | 91.15% |
| 0.57 | 11.15% | 88.84% |
| 0.28 | 12.69% | 87.30% |
| 0.14 | 15.38% | 84.61% |

From the result it can be concluded that when the concentration is 4.6 μM then viable count is 7.30% and the non viable count is 92.69%. On the other hand when the concentration is 0.14 μM the viable count is 15.38% and non viable count is 84.61%. So when the concentrations of Nitazoxanide are increased then the viable count of *Entamoeba histolytica* are decreased and ultimately the non viable count of *Entamoeba histolytica* increased when the concentrations of the drug are increased. It show that Nitazoxanide having antiamoebic activity.

### 6.1.10 viable count of *Entamoeba histolytica* after 24 hours incubation:

**Table 6.8 Viable** counts and it's percentages of *Entamoeba histolytica* after 24h lucubration when the initial count is 5.5 $\times 10^5$ $mL^{-1}$

| Concentration (μM) | Viable parasites count ($mL^{-1}$) | Percentages of viable count of *Entamoeba histolytica* |
|---|---|---|
| 4.6 | $5.5\times10^4$ $mL^{-1}$ | 10% |
| 2.3 | $6.5\times10^4$ $mL^{-1}$ | 11.81% |
| 1.15 | $6.75\times10^4$ $mL^{-1}$ | 12.27% |
| 0.57 | $7.5\times10^4$ $mL^{-1}$ | 13.63% |
| 0.28 | $8.75\times10^4$ $mL^{-1}$ | 15.90% |
| 0.14 | $9.75\times10^4$ $mL^{-1}$ | 17.72% |

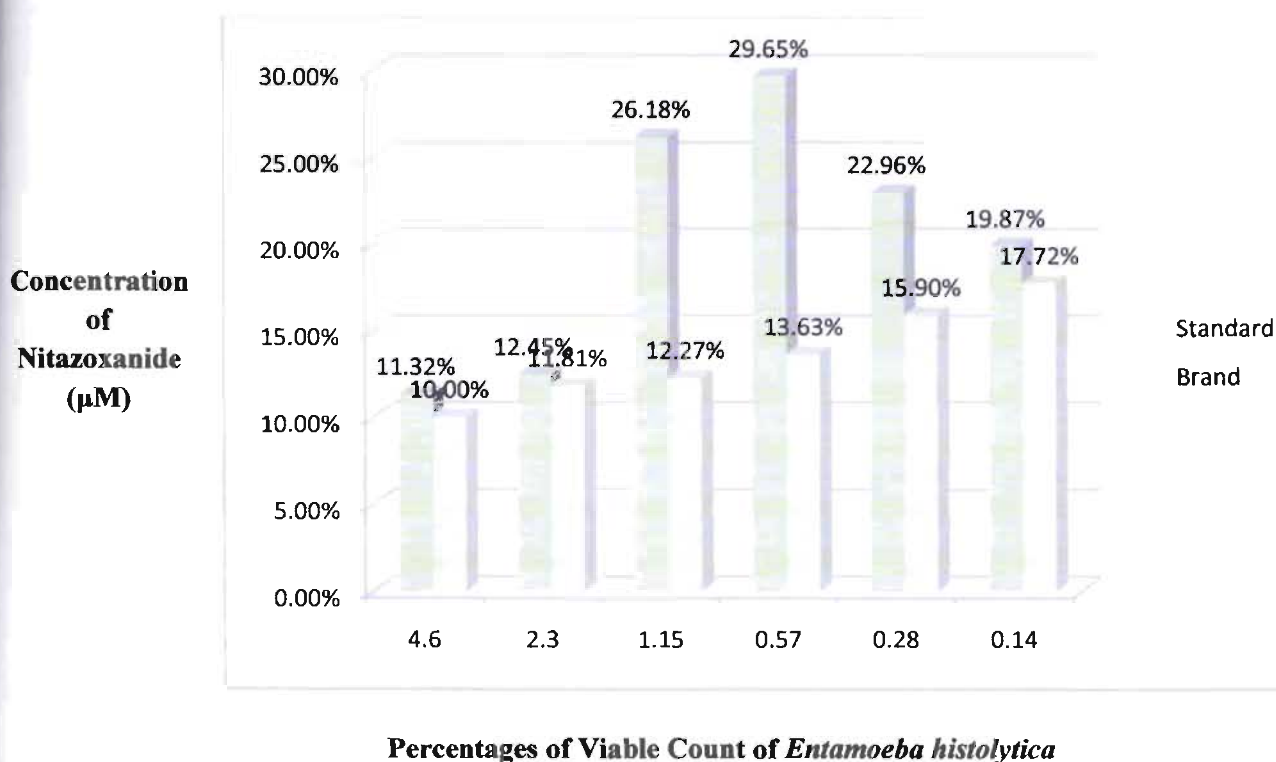



Figure 6.8 Percentages of Viable Counts of the viable counts of *Entamoeba histolytica* after 24 hours incubation when the initial count is 5.5 $\times 10^5$ $mL^{-1}$

When the preparation was completed then it was incubated in 24 hours with different concentration of Nitazoxanide. The initial count of the parasite were 5.5 $\times 10^5$ $mL^{-1}$ and the initial count of the control media were $6.4\times10^5$ $mL^{-1}$.After 24 hours the viable count of *Entamoeba histolytica* was $9.75\times10^4$ $mL^{-1}$ when the concentration of Nitazoxanide. was 0.14 µM and the viable count of *Entamoeba histolytica* were $8.75\times10^4$ $mL^{-1}$, $7.5\times10^4$ $mL^{-1}$, $6.75\times10^4$ $mL^{-1}$ and $6.5\times10^4$ $mL^{-1}$ when the concentration were 0.28, 0.57, 1.15 and 2.3 µM respectively. The viable count *of Entamoeba histolytica* was decreased to $5.5\times10^4$ $mL^{-1}$. when the concentration of Nitazoxanide was increased to 4.6 µM . The numbers of parasites are increased in the control media it indicates that the numbers of parasite are increased after 24 hours incubation. So, the percentages of the viable counts are increased when the concentrations of Nitazoxanide. are decreased.

### 6.1.11 Percentages of Non Viable counts of *Entamoeba histolytica* when the initial count is 5.5 $\times 10^5$ $mL^{-1}$

**Table 6.9** Percentages of the Non Viable counts of *Entamoeba histolytica* when the initial count is 5.5 $\times 10^5$ $mL^{-1}$

| Concentration (µM) | Non Viable parasites count ($mL^{-1}$) | Percentages of non viable count of *Entamoeba histolytica* |
|---|---|---|
| 4.6 | $4.95\times10^5$ $mL^{-1}$ | 90% |
| 2.3 | $4.85\times10^5$ $mL^{-1}$ | 88.18% |
| 1.15 | $4.82\times10^5$ $mL^{-1}$ | 87.72% |
| 0.57 | $4.75\times10^5$ $mL^{-1}$ | 86.36% |
| 0.28 | $4.62\times10^5$ $mL^{-1}$ | 84.09% |
| 0.14 | $4.52\times10^5$ $mL^{-1}$ | 82.27% |

The initial count of the parasites was $5.5\times10^5$ $mL^{-1}$ and the initial count of the control media were $6.4\times10^5$ $mL^{-1}$. After 24 hours the non viable count *of Entamoeba histolytica* were $4.52\times10^5$ $mL^{-1}$ when the concentration of Nitazoxanide was 0.14 µM and the non viable count of *E. histolytica* were $4.62\times10^5$ $mL^{-1}$, $4.75\times10^5$ $mL^{-1}$, $4.82\times10^5$ $mL^{-1}$and $4.85\times10^5$ $mL^{-1}$when the concentration were 0.28, 0.57, 1.15 and 2.3 µM respectively. The non viable count of *Entamoeba histolytica* was increased to $4.95\times10^5$ $mL^{-1}$when the concentration of Nitazoxanide was increased to 4.6 µM . The numbers of parasites are increased in the control it indicates that the numbers of parasite are increased after 24 hours incubation. So, the percentages of the non viable count are increased when the concentration of Nitazoxanide are increased.

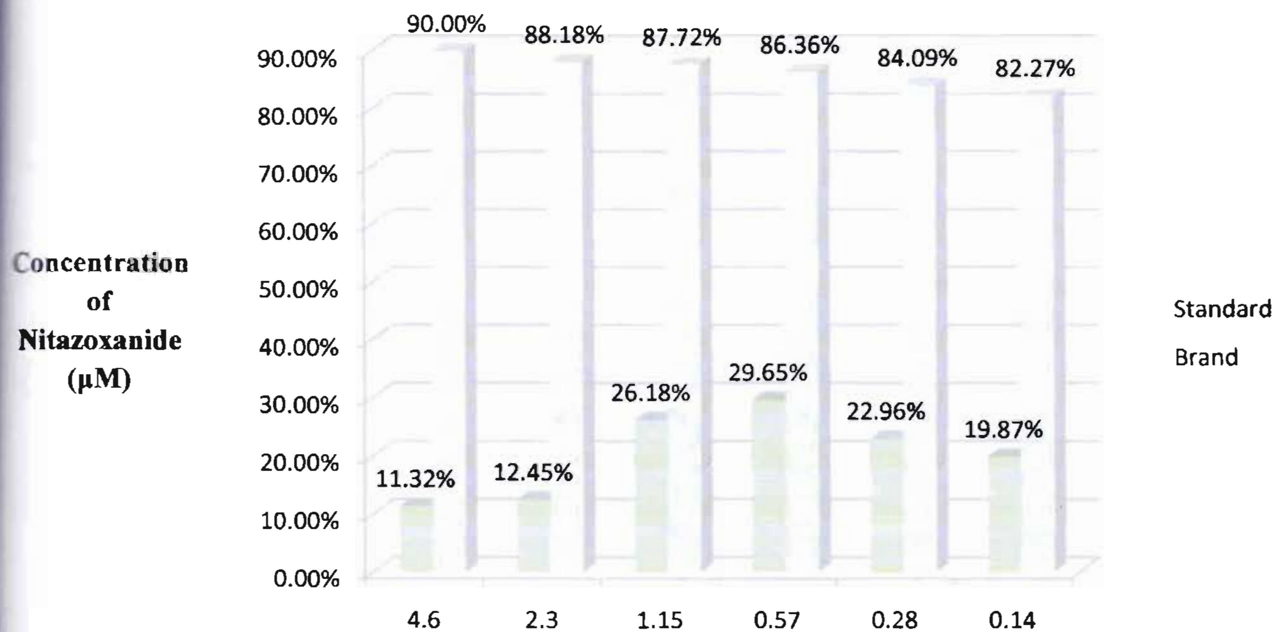



Figure 6.9 Percentages of non viable Counts of the viable counts of *Entamoeba histolytica* after 24 hours incubation when the initial count is $5.5 \times 10^5$ $mL^{-1}$

### 6.1.9 Percentages of Viable counts Non Viable counts of *Entamoeba histolytica* when the initial count is $6.5 \times 10^5$ $mL^{-1}$

**Table 6.10** Percentages of Viable counts Non Viable counts of *Entamoeba histolytica* when the initial count is $6.5 \times 10^5$ $mL^{-1}$

| Concentration of Nitazoxanide (μM) | Percentage of Viable count of *Entamoeba histolytica* | Percentages of non viable count of *Entamoeba histolytica* |
|---|---|---|
| 4.6 | 10% | 90% |
| 2.3 | 11.81% | 88.18% |
| 1.15 | 12.27% | 87.72% |
| 0.57 | 13.63% | 86.36% |
| 0.28 | 15.90% | 84.09% |
| 0.14 | 17.72% | 82.27% |

From the result it can be concluded that when the concentration is 4.6 μM then viable count is 10%and the non viable count is 90%. On the other hand when the concentration is 0.14 μM the viable count is 17.72% and non viable count is 82.27%. So when the concentrations of Nitazoxanide are increased then the viable counts of Entamoeba histolytica are decreased and ultimately the non viable count of *Entamoeba histolytica* increased when the concentrations of the drug are increased. It show that Nitazoxanide having antiamoebic activity.

# CHAPTER SEVEN

# DISCUSSION

## 7.1 Discussion:

Nitazoxanide and other brands drugs that is used against *Entamoeba histolytica* in associated amoebiasis treatment.

Nitazoxanide, a 5-nitrothiazolyl derivative, is effective in the treatment of a broad range of parasitic infections. In vitro, it is active against several protozoa, including *Cryptosporidium parvum, Blastocystis hominis, Entamoeba histolytica* and *Giardia intestinalis.* 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 and the incidence of amoebic dysentery is reduced in a similar fashion to various synthetic drugs. It having high efficacy on inhibition of clinical isolates of *Entamoeba histolytica* which is the organism that is responsible for amebiasis.

The clinical isolates of *Entamoeba histolytica* were treated with Nitzoxanide at different concentration. The experimental concentrations were 0.07, 0.14, 0.28, 0.58, 1.15, 2.3 and 4.6 μM. A control group was made to measure the change in the viable counts and was put into the ELISA plate. Each ELISA plate now contained different concentration of Nitazoxanide and some amount of *Entamoeba histolytica* (100 micro liters). After that 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.*

When the initial count *of Entamoeba histolytica* is $6.3\times10^5$ $mL^{-1}$then after 24 hours the viable count was 5.15% and non viable count was 94.84%when the concentration of Nitazoxanide was 4.6μM. Viable count *of Entamoeba histolytica* was increased to 16.66% when the concentration of Nitazoxanide was decreased to 0.07.

Then the others viable count were $10..5\times10^4$ $mL^{-1}$, $12.75\times10^4$ $mL^{-1}$, $14.75\times10^4$ $mL^{-1}$, $13\times10^4$ $mL^{-1}$and $6.5\times10^4$ $mL^{-1}$ when the concentration were 0.14, 0.28, 0.57, 1.15, 2.3 and 4.6 μM respectively. After 24 hours the non viable count non viable count of *E. histolytica* were $5.25\times10^5$ $mL^{-1}$, $5.02\times10^5$ $mL^{-1}$, $4.82\times10^5$ $mL^{-1}$, $5.0\times10^5$ $mL^{-1}$ and $5.65\times10^5$ $mL^{-1}$. when the concentration were 0.14, 0.28, 0.57, 1.15, 2.3 and 4.6 μM respectively.

When the initial count of *Entamoeba histolytica* is 7.1 $\times10^5$ $mL^{-1}$.After 24 hours the viable count was 6.33% and non viable was 93.66%at 4.6 μM concentration. Viable count is increased to 12.67%and non viable count decreased to 87.32%when the concentration decreased to 0.14

So, the percentages of the non viable counts are increased when the concentration of Nitazoxanide are increased. A control group was made to measure the change in the viable counts and in the non viable counts *of Entamoeba histolytica. The* lowest number of non viable count of *Entamoeba histolytica* was found in the control group and highest in the 4.6 μM concentration of Nitazoxanide In compare to the previous study about "*In Vitro* Effect of Nitazoxanide and other different brand Nitazoxanide against *Entamoeba histolytica, Giardia intestinalis* and *Trichomonas vaginalis* Trophozoites" from Journal of Eukaryotic Microbiology the result of inhibition of *Entamoeba histolytica* are similer. Because about 100% inhibition of growth *of Entamoeba histolytica* was occurred when the concentration of Nitazoxanide is more than 4.6μM.

So, Nitazoxanide and different brands 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. The findings of this study are also helpful to make awareness of both physicians and consumers to select the right drug.

# CHAPTER EIGHT

# CONCLUSION

## 8.1 Conclusion:

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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.

*Entamoeba histolytica.* Amebiasis is the third leading parasitic cause of morbidity and mortality due to parasitic disease in human after malaria and schistomiasis. On a global basis, amebiasis affects approximately 50 million persons each year, resulting in nearly 100,000 deaths.

Nitazoxanide was treated with different concentration and same amount of *Entamoeba histolytica* .The concentrations were 0.07, 0.14, 0.28, 0.57, 1.15, 2.3 and 4.6 μM. All concentrations are used and measured their capability of inhibiting the clinical isolated of *Entamoeba histolytica* and control was used to count the viable parasite without any drug. The rate of inhibition of the clinical isolates increased with the increasing concentration of the drug. The maximum concentration of Nitazoxanide was 4.6 μM which results about 100% inhibition of the clinical isolates of *Entamoeba histolytica* for 24 hours incubation period.
From the study we can found that Nitozoxanide and different brands are effective against *Entamoeba histolytica* which is responsible for amebiasis. So, Nitazoxanide is a good treatment option for the patient who suffering from amebiasis.
So, from the above study we can concluded that Nitazoxanide and other different brands are effective in the treatment of a broad range of parasitic infections and the in vitro sensitivity of Nitazoxanide against clinical isolates of *Entamoeba histolytica* is very high.

# CHAPTER NINE

# REFERENCES

## 9.1 REFERENCES


Berger SA, Marr JS (2006). *Human Parasitic Diseases Sourcebook.* Jones and Bartlett Publishers: Sudbury, Massachusetts,

Visvesvara GS, Moura H, Schuster FL (June 2007). "Pathogenic and opportunistic free-living amoebae: Acanthamoeba spp., Balamuthia mandrillaris, Naegleria fowleri, and Sappinia diploidea". *FEMS Immunol. Med. Microbiol.* **50** (1): 1–26.

Day, David W.; Basil C. Morson, Jeremy R. Jass, Geraint Williams, Ashley B. Price (2003). *Morson and Dawson's Gastrointestinal Pathology*. John Wiley & Sons, Inc.

Brock Biology of Microorganisms; Madigan (et al.); Pearson Education Inc., 2003; page. 947-948

Ryan KJ, Ray CG (editors) (2004). *Sherris Medical Microbiology* (4th ed.). McGraw Hill. pp. 733–8.

"Amoebiasis". *Wkly. Epidemiol. Rec.* **72** (14): 97–9. April 1997. PMID 9100475. American Water Works Association (2006-06). *Waterborne Pathogens*

Stanley SL (March 2003). "Amoebiasis". *Lancet* 361 (9362): 1025–34. doi:10.1016/S0140-6736(03)12830-9. PMID 12660071

Bhattacharya A, Ghildyal R, Prasad J, Bhattacharya S and Diamond L S 1992a *Modulation of a surface antigen of Entamoeba histolytica in response to bacteria;* Infect. Immun. 60 1711-1713

Adams EB, MacLeod IN. Invasive amebiasis. II. *Amebic liver abscess and its complications.* Medicine (Baltimore) 1977.

Am. J. Trop. Med. Hyg. American Water Works Association (2006-06). *Waterborne Pathogens..*, 68(4), 2003,

Amebiasis. Available from: http://www.adam.com/ency/article/000298trt.htm. Accessed 2/25/00

Berger SA, Marr JS. *Human Parasitic Diseases Sourcebook.* Jones and Bartlett Publishers: Sudbury, Massachusetts, 2006.

Baron S, 4th edition.Galveston (TX): University of Texas Medical Branch at Galveston; 1996.

Boeck, W. C.? and J. Drbohlav. 1925. The cultivation *ofEndamoeba histolytica.* Am. J. Hyg. 5:371-407.

Balamuth, W. 1946. Improved egg yolk medium for cultivation *ofEntamoeba histolytica* and other intestinal protozoa. Am. J. Clin, Pathol. 16:380-384.

Bad Bug Book, Entamoeba histolytica. Available From:
http://vm.cfsan.fda.gov/~mow/chap23.html. Accessed 3/02/00 Bug Bytes Volume 2 Number 12-July 20, 1995. Available from: http://www.ccm.lsumc.edu/bugb)^tes/Volume2/bb-v2nl2.htm. Accessed 3/05/00

Beck DL, Tanyuksel M, Mackey AJ, et al. *Sequence conservation of the Gal/GalNAc lectin from clinical isolates.* Exp Parasitol 2002

Caler, E and Lorenzi, H (2010). "*Entamoeba histolytica:* Genome Status and Web Resources". *Anaerobic Parasitic Protozoa: Genomics and Molecular Biology.*

Cleveland, L. R., and E. P. Sanders. 1930. *The production of bacteria-free amoebic abscesses in the liver of cats and observations on the amoebae in various media with and without bacteria.* Science. 77:149-151.

Diamond, L. S. 1968. Techniques of axenic cultivation *ofEntamoeba histolytica* Schaudinn, 1903 and *E. histolytica-like* amebae. J. Parasitol. 54:1047-1056.

Diamond, L. S. 1968. Techniques of axenic cultivation *ofEntamoeba histolytica* Schaudinn, 1903 and *E. histolytica-\\ke* amebae. J. Parasitol. 54:1047-1056. & Diamond, L. S. 1961. Axenic cultivation *ofEntamoeba histolytica.* Science. 134:336-337.XX

Diamond, L. S., D. R. Harlow, and C. C. Cunnick. 1978. A new medium for the axenic cultivation *ofEntamoeba histolytica* and other *Entamoeba.* Trans. R. Soc. Trop. Med. Hyg. 72:431-432.

Diamond, L. S., C. G. Clark, and C. C. Cunnick, 1995. YI-S, a casein-free medium for axenic cultivation *ofEntamoeba histolytica,* related *Entamoeba, Giardia intestinalis* and *Trichomonas vaginalis.* J. Euk. Microbiol. 42:277-278.

Diamond, L. S, 1982. A new liquid medium for xenic cultivation *ofEntamoeba histolytica* and other lumen dwelling protozoa. J. Parasitol. 68:958-959.

*Entamoeba histolytica* .Ravdin,JI.(Ed).John Wiley and Sons, New York,pp:93-105. Friedman, Lawrence S. "Liver, Biliary Tract, & Pancreas." In *Current Medical Diagnosis and Treatment, 1998,* edited by Stephen McPhee, et al., 37th ed. Stamford: Appleton & Lange, 1997.

Fotedar R, Stark D, Beebe N, et al. Laboratory diagnostic techniques for Entamoeba species. *Clin Microbiol Rev,* Jul 2007;20(3):511-32, table of contents.

Gillin, F. D., and L. S. Diamond 1978. Clonal growth *ofEntamoeba histolytica* and other species *ofEntamoeba* in agar. J. Protozool. 25:539-543.

Health Canada Notifiable Diseases on Line-Amoebiasis. Available from: http://cythera.ic.gc.ca/spansweb/ndis/diseases/anioe_e.html. Accessed 3/05/00

Hollander, D. H. 1976. Colonial morphology of *Trichomonas vaginalis* in agar. J. Parasitol. 62:826-828

Ho, L. C., M. Singh, G. Suresh, G. C. Ng, and E. H. Yap. 1993. Axenic culture of *Blastocystis hominis* in Iscove's modified Dulbecco's medium. Parasitol. Res. 79:614-616

Clark, C. G. and L. S. Diamond 2002. Methods for cultivation of luminal parasitic protists of clinical importance. Clin. Microbiol. Rev. 15: 329-341

Childhood Infections-Amebiasis: Available from: http:// kidshealth.org/parent/ common/ amebiasis.html. Accessed 3/05/00

Centers for Disease Control and Prevention, Parasitic Disease Information, Fact Sheet. Available from: http://www.cdc.gov/ncidod/dpd/parasites/amebiasis/factsht_amebiasis.htm. Accessed 02/25/00

Dialogue on Diarrhoea. Issue no. 27-December 1986. Available from: http://www.ehydrate.org/dd/html/dd27.htm. Accessed 3/02/00

Day, David W.; Basil C. Morson, Jeremy R. Jass, Geraint Williams, Ashley B. Price (2003). *Morson and Dawson's Gastrointestinal Pathology.* John Wiley & Sons, Inc

Diamond, L. S. 1983. Lumen dwelling protozoa: *Entamoeba,* trichomonads, and *Giardia,* p. 67-109. *In* J. B. Jensen (ed.)s *In vitro* cultivation of protozoan parasites. CRC Press, Boca Raton, FL.

Diamond, L. S. 1983. Lumen dwelling protozoa: *Entamoeba.,* trichomonads, and *Giardia,* p. 67-109. *In* J. B. Jensen (ed.), *In vitro* cultivation of protozoan parasites. CRC Press, Boca Raton, FL.

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Diamond, L. S. 1983. Lumen dwelling protozoa: *Entamoeba,* trichomonads, and *Giardia,* p. 67-109. *In* J. B. Jensen (ed.), *In vitro* cultivation of protozoan parasites. CRC Press, Boca Raton, FL.

Dobell, C., and P. P. Laidlaw. 1926. On the cultivation *of Entamoeba histolytica* and some other entozoic amoebae. Parasitology. 18:283-318.3. Dobell, C., and P. P. Laidlaw. 1926.

On Dougherty, E. C. 1959. Introduction to axenic cultivation of invertebrate metazoa: a goal. Ann. N.Y. Acad. Sci. 77:27-54. http://www.dpd.cdc.gov/DPDx/HTML/Search_Choices.htm. Accessed 3/04/00

Robinson, G. L. 1968. The laboratory diagnosis of human parasitic amoebae. Trans. R. Soc. Trop. Med. Hyg. 62:285-294.

Ryan KJ, Ray CG (editors) (2004). *Sherris Medical Microbiology* (4th ed.). McGraw Hill.

Rossignol JF, La Frazia S, Chiappa L, Ciucci A, Santoro MG. Thiazolides, a new class of anti-influenza molecules targeting viral hemagglutinin at the post-translational level. J Biol Chem,2005.

Robinson, G. L. 1968. The laboratory diagnosis of human parasitic amoebae. Trans. R. Soc. Trop. Med. Hyg. 62:285-294.

Reeves, R. E., H. E. Meleney, and W. W. Frye. 1957. A modified Shaffer-Frye technique for the cultivation *ofEntamoeba histolytica* and some observations on its carbohydrate requirements. Am. J. Hyg. 66:56-62.

Smedley, S. R. 1956. A method for freeing cultures *ofEntamoeba histolytica* from contamination with *Blastocystis.* Trans. R. Soc. Trop. Med. Hyg. 50:232-23 Stanley SL Jr. Amoebiasis. *Lancet* Mar 22 2003.

Sapna Gupta, Vishal R. Tandon, Sunil Dutt Sharma, Ritu Gupta, Ravinder K. Gupta 2001 Seydel KB, Li E, Zhang Z, Stanley SL. Epithelial cell-initiated inflammation plays a crucial role in early tissue damage in amebic infection of human intestine. Gastroenterology 1998

Stanley SL (March 2003). "Amoebiasis". *Lancet* 361 (9362): 1025-34. vanSonnenberg E. Mueller PR, Schiffinan HR, et al. Intrahepatic amebic abscesses: indications for and results of percutaneous catheter drainage. Radiology 1985

Von Brand, T., C. R. Rees, L. Jacobs, and L. V. Reardon. 1943. Studies on reducing substances and gas formation in cultures *ofEndamoeba histolytica* and a single species of symbiotic bacterium. Am. J. Hyg. 37:310-319.

World Journal of Gastroenteroiogy 2009 available at
http://www.dpd.cdc.gov/DPDx/HTML/Search_Choices.htm. Accessed 3/12/10 WHO, 1997 .Amoebiasis. Wkly .Epidemiol.Rec.,72(14):97-99.

*Wldy. Epidemiol Rec.* 72 (14): 97-9. April 1997 available at http://www-micro.msb.le.ac.uk/224/Parasitol.html. Accessed 3/04/00

Walsh, A.L, .1988. Prevalence in *Entamoeba histolytica* infection. In: Amoebiasis http://www.dpd.cdc.gov/DPDx/HTML/Search_Choices.htm. Accessed 3/09/10

Zierdt, C. H., and R. L. Williams. 1974. *Blastocystis hominis:* axenic cultivation. Exp. Parasitol. 36:233-243.