In vitro Sensitivity Test of Metronidazole by Using Clinical Isolates of *E. histolytica* and *E. bangladeshi*

A Dissertation Submitted to the Department of Pharmacy, East West University, as the Partial Fulfillment of the Requirements for the Degree of Master of Pharmacy.

Submitted by

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Date of Submission: 16.07.2017

Declaration by the Research Candidate

I, Evana Afrose, hereby declare that this dissertation entitled "*In vitro* Sensitivity Test of Metronidazole by Using Clinical Isolates of *E. histolytica* and *E. bangladeshi*" submitted to the Department of Pharmacy, East West University, in partial fulfillment for the requirement of the Degree of Master of Pharmacy, is an authentic research work done by me under the guidance of Professor Dr. Sufia Islam, Department of Pharmacy, East West University, Dhaka Bangladesh. The content of this dissertation in full or in parts, have not been submitted to any other Institution or University for the award of any Degree or any Diploma of Fellowship.

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Certificate by the Supervisor

This is to certify that the dissertation, entitled "*In vitro* Sensitivity Test of Metronidazole by Using Clinical Isolates of *E. histolytica* and *E. bangladeshi*" is a bonafide research work done under our guidance and supervision by Evana Afrose (ID # 2015-3-79-011), in partial fulfillment for the requirement of the Degree of the Master of Pharmacy.

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Certificate by the Chairperson

This is to certify that the dissertation, entitled "*In vitro* Sensitivity Test of Metronidazole by Using Clinical Isolates of *E. histolytica* and *E. bangladeshi*" is a bona fide research work done by Evana Afrose (ID # 2015-3-79-011), in partial fulfillment for the requirement of the Degree of the Master of Pharmacy.

Prof. Dr. Chowdhury Faiz Hossain

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Dedication

This research paper is dedicated to my

Beloved Parents

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Abstract

Infectious diseases remain the major cause of morbidity and mortality worldwide including Bangladesh. *Entamoeba histolytica* is the etiological agent of amoebic dysentery and amoebic liver abscess (ALA). Worldwide, 40–50 million symptomatic cases of amoebiasis occur annually and 70,000 to 100,000 deaths are due to this infection. In amoebiasis Metronidazole, a 5-nitroimidazole drug is the drug of choice for intestinal disease caused by *Entamoeba histolytica*. In 2010-2011 a new species of *Entamoeba* named *E. bangladeshi* has been identified, which was positive for *Entamoeba* organism but negative for *E. histolytica*, *E. dispar* and *E. moshkovskii* by PCR. It was found in the feces of children living in Mirpur, Dhaka, Bangladesh. Till now no studies have been carried out to determine the sensitivity of Metronidazole against this new species. Therefore, the objective of the study is to determine the sensitivity of metronidazole against clinical isolate of *E. bangladeshi*. The clinical isolates of *E. histolytica* and *E. bangladeshi* were treated with metronidazole at different concentrations (1.28, 0.64, 0.32, 0.16, 0.08, and 0.04 µg/ml). Drug sensitivity assay of the samples was carried out by using microtiter plates containing 100 µg/ml of parasite suspension (1×10^6 parasites/ml). Plates were incubated at 37^0C. After 4 hours incubation the viable parasites were counted by haemocytometer under the microscope. Viable counts of the *E. histolytica* and *E. bangladeshi* in each concentration of drugs were compared to the control. Result showed that viable cell count is significantly reduced in the treatment groups when compared with the control ($p=<0.05$). However, the viable cell count of *E. bangladeshi* is similar when it was compared with cell count of *E. histolytica* and no significant difference has been observed between these two groups. We have also found that inhibition of parasite is occurred in a dose dependent manner. Cell inhibition is maximum at the highest concentration of Metrondazole. The cell inhibition is 32% and 18% when Metronidazole treated against *E. histolytica* and *E. bangladeshi* respectively. We can conclude that treatment with Metronidazole drug may be a useful treatment to inhibit the growth of *E. bangladeshi*. Further study is needed to explore different drugs at different concentrations and time intervals to determine the *in vitro* sensitivity against *E. bangladeshi* isolates.

Introduction

1.1 Background

Amoebiasis is a disease caused by the parasite *Entamoeba histolytica*. Hippocrates who described a patient with fever and dysentery first recognized it as a deadly disease. With the application of a number of new molecular biology-based techniques, tremendous advances have been made in our knowledge of the diagnosis, natural history, and epidemiology of amoebiasis. On a global basis, amoebiasis affects approximately 50 million persons each year resulting in 100,000 deaths (Petri et al., 2003). Amoebiasis is also very common in Bangladesh. Developments came later in 1855 when it was suggested that the disease might have a parasitic origin. Finally, *E. histolytica* was identified from a stool sample in 1875 by Fredor Losch. *Entamoeba histolytica* is the etiological agent of amoebic dysentery. *E. histolytica* is transmitted via ingestion of the cystic form (infective stage) of the protozoa. Viable in the environment for weeks to months, cysts can be found in fecally contaminated soil, fertilizer, or water or on the contaminated hands of food handlers (Walsh et al., 1986). Recently there was an study published which discovered a strain named *Entamoeba bangladeshi* which can be distinguished visually from other members of the *Entamoeba* genus and also a reason for amoebiasis (Royer et al., 2012). Throughout the world, amoebiasis is the second leading cause of death from a parasitic disease. Although it is the second leading cause of death from parasitic diseases, about 90% of the people exposed to *E. histolytica* are asymptomatic or report very mild symptoms (Tanyuskel et al., 2003).

1.2 Amoebiasis

Amoebiasis is a condition in which intestines (gut) becomes infected with the parasite *E. histolytica*. *Entamoeba* is a group of single-celled parasites (living things that live in, or on, other living organisms) that can infect both humans and some animals. Amoebiasis can

present with no, mild, or severe symptoms. Symptoms may include abdominal pain, mild diarrhoea, bloody diarrhea or severe colitis with tissue death and perforation. Most people who become infected with *E. histolytica* do not develop any symptoms. However, symptoms may develop if the parasite causes inflammation of the lining of gut. In some people, *E. histolytica* can also get into the bloodstream from the gut and spread around the body to the liver, lungs and sometimes other organs. Amoebiasis remains an important health problem in tropical countries where sanitation infrastructure and health are often inadequate. Only about 10% to 20% of people who are infected with *E. histolytica* become sick from the infection. It can affect anyone, although it is more common in people who live in tropical areas with poor sanitary conditions *(*Farrar et al., 2013).

Amoebiasis is a common infection of the human gastro-intestinal tract. Amoebiasis is more closely related to poor sanitation and socioeconomic status than to climate. It has worldwide distribution. It is a major health problem in China, South East and West Asia and Latin America, especially Mexico and Bangladesh (Sherris Medical Microbiology (4th ed.). In 1969, WHO defined amoebiasis, a condition in which a patient harbouring the organism *Entamoeba histolytica* in the bowel. Culture of *E. histolytica* is a long and laborious process. There are three basic types of culture systems of *E. histolytica,* xenic, in which the parasite is grown in the presence of an undefined flora; monoxenic, in which the parasite is grown in the presence of a single additional species; and axenic in which the parasite is grown in the absence of any other metabolizing cells (Weedall and Hall, 2011).

1.3 Amoeba

The word amoeba comes from a Greek word meaning, "to change." An amoeba is any of several tiny, one-celled protozoa in the phylum (or primary division of the animal kingdom) Sarcodina. Amoebas live in freshwater and salt water, in soil, and as parasites in moist body parts of animals. They are composed of cytoplasm (cellular fluid) divided into two parts: a thin, clear, gel-like outer layer that acts as a membrane (ectoplasm); and an

inner, more watery grainy mass (endoplasm) containing structures called organelles. Amoebas may have one or more nuclei, depending upon the species. The well-known type species, Amoeba proteus, is found on decaying bottom vegetation of freshwater streams and ponds (UXL Encyclopedia of Science, 2002).

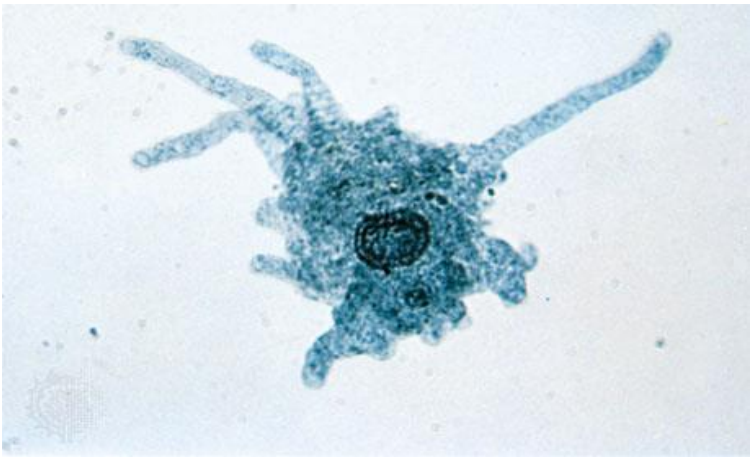

Figure 1.1: Amoeba

There are many varieties of free-living amoeba, but only four genera have been causally associated with disease in humans such as *E. histolytica* is the cause of amoebiasis, or amoebic dysentery, *Naegleria fowleri* (the "brain-eating amoeba") is a fresh-water-native species that can be fatal to humans if introduced through the nose. *Acanthamoeba* can cause amoebic keratitis and encephalitis in humans, *Balamuthia mandrillaris* is the cause of (often fatal) granulomatous amoebic meningoencephalitis. They are distinct from the more famous *Entamoeba histolytica* (an obligate anaerobic parasite which can cause amoebiasis, amoebic dysentery and amoebic liver abscesses). *Acanthamoebas* and *B. mandrillaris* are opportunistic pathogens causing infections of the CNS, lungs, sinuses and skin, mostly in immune compromised humans. *B. mandrillaris* is also associated with disease in immune competent children, and *Acanthamoeba*s pp. cause a sight-threatening keratitis, mostly in contact lens wearers. *N. fowleri* causes an acute and fulminating meningoencephalitis in immune competent children and young adults *(*Jeon, Kwang, 1973).

1.4 Amoeba and human disease

There are at least six species of amoeba, in the broader sense of amoebozoa, which are parasitic in humans. However, most of them have an impact on the body characterized as "nonpathogenic intestinal amoeba infection." That means that these parasites are classified as ones that never make people sick or harm the body, even in people with weak immune systems. For example, *Entamoeba coli*, *Entamoeba dispar*, and *Entamoeba hartmanni* are considered harmless parasites.

Entamoeba histolytica, however, is a parasitic amoebozoa that negatively impacts the human body, resulting in the infectious disease known as amebiasis. It is most common in developing countries that have poor sanitary conditions. Mild amoebiasis is associated with stomach pain and stomach cramping, but a severe form is amoebic dysentery, which can cause stomach pain, fever, and bloody stools. In the worse cases, it can spread to other parts of the body, including the brain and lungs (UXL Encyclopedia of Science, 2002).

1.5 *Entamoeba histolytica*

Entamoeba histolytica is an anaerobic parasitic amoebozoa, part of the genus *Entamoeba*. There are at least six species of *Entamoeba* that can infect the human gut but only *E. histolytica* causes disease. Predominantly infecting humans and other primates causing amoebiasis, *E. histolytica* is estimated to infect about 50 million people worldwide. *Entamoeba histolytica* is the etiological agent of amoebic dysentery and amoebic liver abscess (ALA). Worldwide, 40–50 million symptomatic cases of amoebiasis occur annually and 70,000 to 100,000 deaths due to this infection (Walsh JA et al., 1968). *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 per cent of the infections. Poverty, ignorance, overcrowding, poor sanitation and malnutrition favor transmission and increased disease burden. Prevalence varies from country to country and within a country (Lauren, 2016).

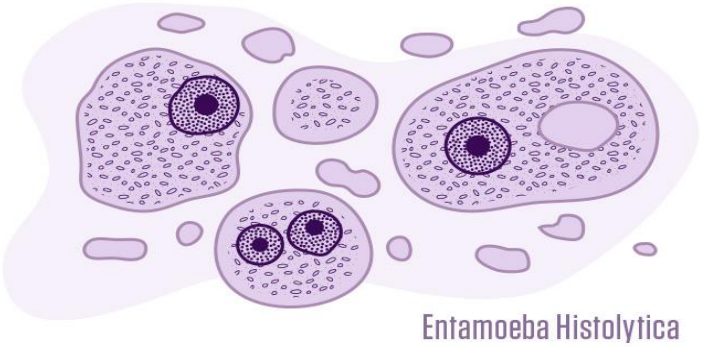

Fig: 1.2. Microscopic image of *E. histolytica*

E. histolytica infections can be detected through fecal microscopy, culture, PCR, and antigen detection.

1.6 Species of *Entamoeba*

The genus *Entamoeba* contains many species, six of which (*Entamoeba histolytica, Entamoeba dispar, Entamoeba moshkovskii, Entamoeba polecki, Entamoeba coli*, and *Entamoeba hartmanni*) reside in the human intestinal lumen. *Entamoeba histolytica* is the causative agent of amebiasis and is considered a leading parasitic cause of death worldwide in humans. Although recent studies highlight the recovery of *E. dispar* and *E. moshkovskii* from patients with gastrointestinal symptoms, there is still no convincing evidence of a causal link between the presence of these two species and the symptoms of the host (Ryan & Ray, 2004). New approaches to the identification of *E. histolytica* are based on detection of *E. histolytica* -specific antigen and DNA in stool and other clinical samples. Several molecular diagnostic tests, including conventional and real-time PCR, have been developed for the detection and differentiation of *E. histolytica*, *E. dispar*, and *E. moshkovskii* in clinical samples (Fotedar et al., 2007).

1.7 Scientific classification of *E. histolytica*

Kingdom	Protista
Subkingdom	Protozoa
Phylum	Sarcomastigophora
Class	Lobosea
Order	Amoebida
Family	Entamoebidae
Genus	*Entamoeba*
Species	Histolytica

1.8 Distribution of *E. histolytica*

E. histolytica is cosmopolitan in distribution, but is more common in tropical and sub-tropical countries. In India it occasionally takes an epidemic form. It is estimated that about seven to eleven per cent of the population in India suffers from its infection. The prevalence of *Entamoeba* infection is as high as 50% in areas of Central and South America, Africa, and Asia. *E. histolytica* seroprevalence studies in Mexico revealed that more than 8% of the populations were positive (Caballero-Salcedo, 1994). In endemic areas, as many as 25% of patients may be carrying antibodies to *E. histolytica* as a result of prior infections, which may be largely asymptomatic. The prevalence of asymptomatic *E. histolytica* infections seem to be region-dependent; in Brazil, for example, it may be as high as 11% (Tengku SA, Norhayati M, 2011).

1.9 Life cycle of *E. histolytica*

E. histolytica is a monogenetic parasite as its life cycle is completed in a single host i.e., man. Three distinct morphological forms exist in its life cycle. -Trophozoite, Pre-cystic stage and Cystic stage (Sherris Medical Microbiology (4th ed.).

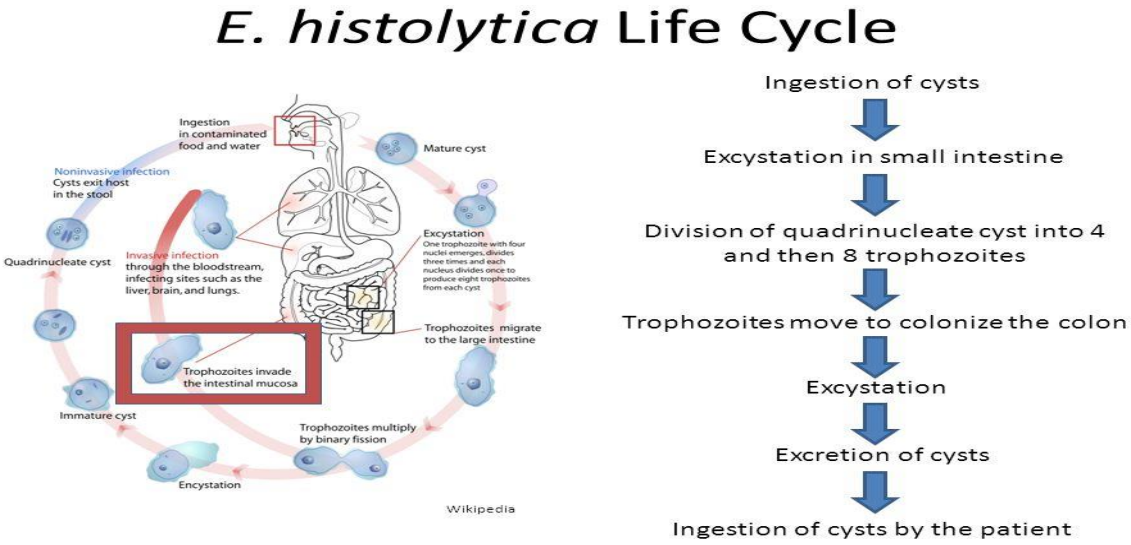

Figure 1.3: Life cycle of *E. histolytica*

1.10 Stages of *E. histolytica*

The life cycle of *Entamoeba histolytica*, includes three stages ,

1. Trophozoite stage,
2. Precystic stage and
3. Cystic stage.

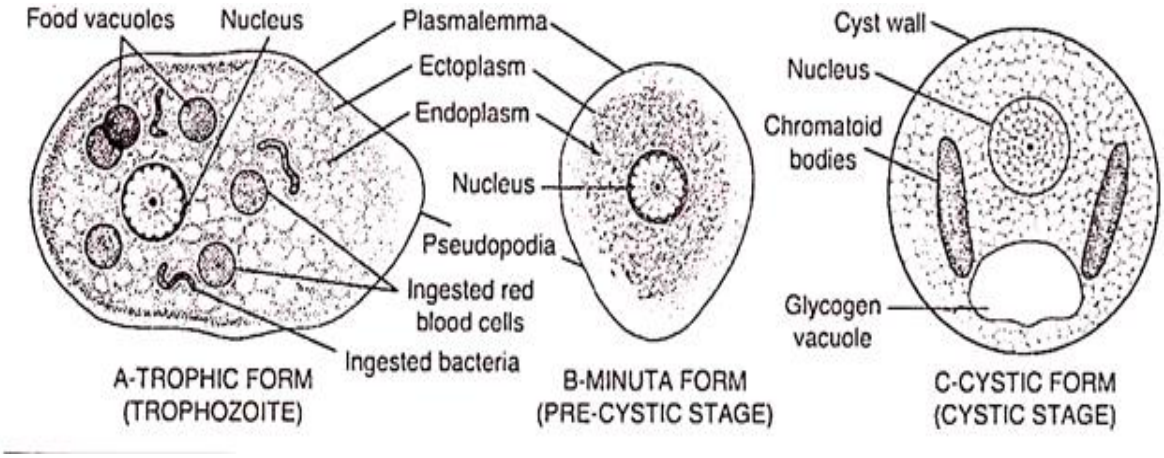

Figure 1.4: Stages of *E. histolytica*

1. **Trophozoite stage**

Trophozoite is the motile, feeding and pathogenic stage of *E. histolytica*. It measures about 20-30 microns in diameter. Trophozoite is surrounded by the limiting membrane called plamalemma (William and Sodeman, 1996).

2. **Precystic stage**

Some of the daughter amoebae that are entered into the lumen of intestine develop transform into precystic stage. It is the nonmotile , nonfeeding and nonpathogenic stage of *E. histolytica* . It is relatively smaller in size, measuring about 10-20 micrometers (William and Sodeman, 1996).

3. **Cystic stage**

It is found in the lumen of the large intestine. It is round in shape and is surrounded by a thin, delicate and highly resistant wall. The precess of development of cyst wall is called encystations which is a means to tide over the unfavorable conditions that the parasite is going to encounter while that the parasite is going to encounter while passing to a new host (William and Sodeman, 1996).

1.11 Mode of infection

A matured quadrinucleate cyst of *E. histolytica* is the infective stage of the parasite. Transmission of *E. histolytica* from one person to another occurs due to ingestion of these cysts. Fecal contamination of edible substances and drinking water are the primary cause of infection. Following are the mode of transmission of this parasite.

(a) Fecal-oral route

In majority of cases infection takes place through intake of contaminated uncooked vegetables and fruits. Insect vectors like flies, cockroaches and rodents act as agent to carry infective cysts to the food and drink. Sometimes drinking water supply contaminated with infected faces gives rise to epidemics (Saritha, 2015).

(b) Oral-rectal contact

Sexual transmission by oral-rectal contact is also one of the modes of transmission, especially among male homosexuals (Saritha, 2015).

1.12 Excystation

The process of excystation begins when the quadrinucleate cyst enters in the ileum of the small intestine of the new host. Excystation is the process of transformation of cysts to the trophozoites. It occurs in the intestinal lumen of the host. The cyst wall gets dissolved by the neutral or alkaline intestinal juice. The nucleus inside the cyst divides once again to form eight daughter nuclei.

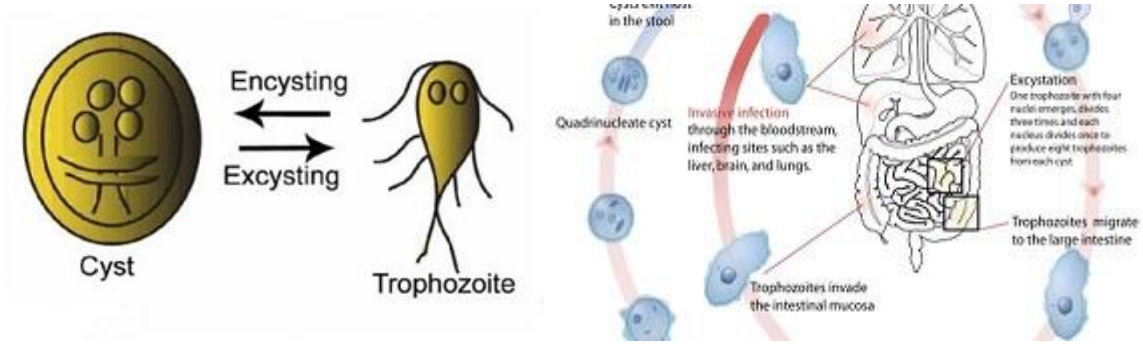

Figure 1.5: Excytation process

Certain amount of cytoplasm surrounds each of the nuclei to form 8 trophozoites. The parasite at this stage moves into the caecum of the host's large intestine, get attached to the epithelial cells of the large intestine, produces necrosis by proteolytic ferment (cytolysin) and enters into the mucosa and sub-mucosa layers by means of their own mobility action (Saritha, 2015).

1.13 Pathophysiology of *E. histolytica*

The lesions produced by *E. histolytica* are primarily intestinal and secondary extra intestinal. The intestinal lesions are confined to the large intestine, frequently cecal and sigmoidorectal regions. The typical flask like primary ulcer to large nacrotic areas is produced. In acute amoebiasis, there are severe dysentery in numerous small stools containing blood, mucous and necrotic mucosa accompanied by acute abdominal pain, tenderness and fever. Chronic amoebiasis is characterized by recurrent attacks of dysentery with gastrointestinal disturbance. In extra intestinal amoebiasis, the liver is invaded chiefly, resulting amoebic hepatitis or liver abscess. An enlarged, tender liver, with pain in upper right hipochondrium, characterizes it. Less frequently, lung abscess, splenic abscess, brain abscess or cutaneous amoebic lesions are seen.

Ingested cysts of *E. histolytica* excyst in the small intestine are showing in figure below. Trophozoites are carried to the colon, where they mature and reproduce. The parasite may lead a commensal existence on the mucosal surface and in the crypts of the colon. Successful colonization depends on factors such as inoculum size, intestinal motility, transit time, the presence or absence of specific intestinal flora, the host's diet and the ability of the ameba to adhere to the colonic mucosal cells. The ameba adherence molecule has been identified as a lectin which can bind to either of two common carbohydrate components of cell membrane, galactose and N-acetyl glactoseamine. Binding to colonic mucins blocks adherence to mucosal cells. Depletion of mucus results in binding to the mucosa, an essential step in the development of the disease. If amebas pass down the colon they encyst under the stimulus of desiccation, and then are evacuated with the stool (William and Petri 1993).

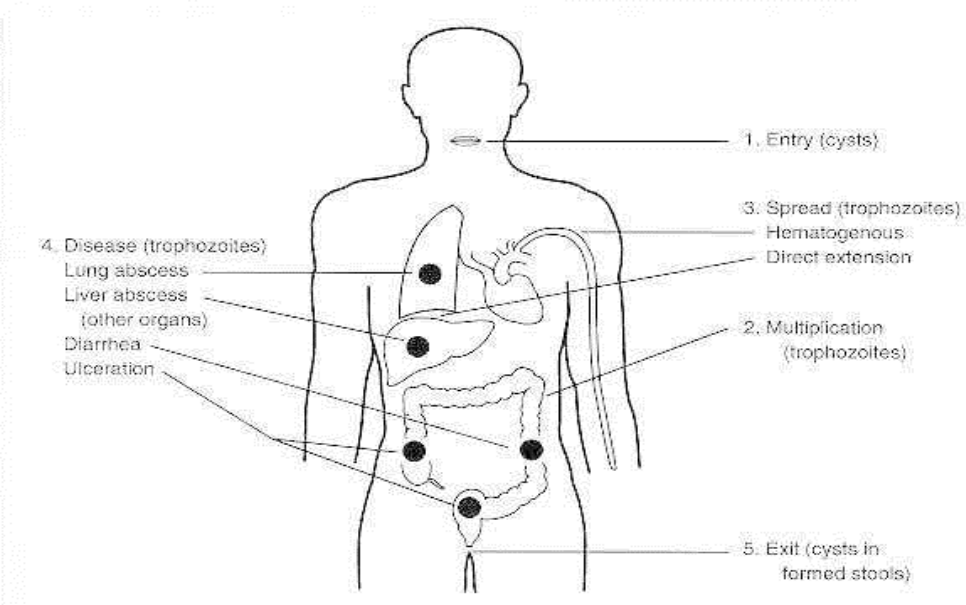

Figure 1.6: Pathophysiology of *E.histolytica*

1.14 *Entamoeba bangladeshi*

Diarrheal diseases have a major effect on global health, particularly the health of malnourished children. The enteric parasites *Entamoeba histolytica* and *E. moshkovskii* are potential causes of diarrheal disease in children. For the past 20 years, it has been studying *Entamoeba* infections in children from the urban slum of Mirpur in Dhaka, Bangladesh. *E. histolytica* infections can be detected through fecal microscopy, culture, PCR, and antigen detection. Microscopy and culture have limited specificity because several species of *Entamoeba*, which vary in their pathogenic potential, have morphologically similar cysts and trophozoites.

In 2010–2011, during analysis of feces which are positive for *Entamoeba* organisms by microscopy or culture but negative for *E. histolytica* , *E. dispar*, and *E. moshkovskii* by PCR, a new species was identified, which have named *Entamoeba bangladeshi* nov. sp. in recognition of the support of the Bangladesh community for this research. Feces from both diarrheal and surveillance specimens were collected from a cohort of children living in Mirpur. A total of 2,039 fecal samples were examined microscopically (0.9% saline smear) and/or by fecal culture for amebic trophozoites and cysts. One hundred forty-nine (7%) of the samples were positive by microscopy and/or culture for an *Entamoeba* parasite with both cysts and trophozoites that closely resembled those of *E. histolytica , E. moshkovskii,* and *E. dispar* (Royer TL, 2012).

By light microscopy, there were no apparent differences between *E. bangladeshi* and *E. histolytica*. The physical resemblance between *E. histolytica* and *E. bangladeshi* is notable because direct microscopic examination of fecal samples is still used as a diagnostic tool in areas to which these species are endemic to detect *E. histolytica* parasites (Haque R, Mondal D, 2009). To further characterize *E. bangladeshi*, it has the ability to established in xenic culture, and it displayed the ability to grow at 37°C and 25°C, a characteristic shared with *E. moshkovskii* and *E. ecuadoriensis* but that distinguishes it from *E. histolytica* and *E.*

dispar. Tests negative in *E. histolytica* ELISA and in species-specific PCRs. Currently only identifiable by its small subunit ribosomal RNA gene sequence (Stensvold CR, 2011).

1.15 Antiamoebic drugs

Infection is primarily treated by instituting antiamoebic therapy. The detection of *Entamoeba histolytica* and *Entamoeba bangladeshi* is an important goal of the clinical microbiology laboratory. This is because amebiasis is presently one of the three most common causes of death from parasitic disease. Drugs of choice for invasive amoebiasis are tissue active agents, like metronidazole, Ornidazole and chloroquine or the more toxic emetine derivatives, including dehydroemetine. Metronidazole is derived from 5-nitroimdazole which kill the trophozoites by alterations in the protoplasmic organelles of the amoeba.

Nitroimidazole therapy leads to clinical response in approximately 90% of patients with mild-to-moderate amebic colitis. Because intraluminal parasites are not affected by nitroimidazoles, nitroimidazole therapy for amebic colitis should be followed by treatment with a luminal agent (eg, paromomycin or diloxanide furoate) to prevent a relapse.

Amebic liver abscess of up to 10 cm can be cured with metronidazole without drainage. Clinical defervescence should occur during the first 3-4 days of treatment. Failure of metronidazole therapy may be an indication for surgical intervention.

Chloroquine has also been used for patients with hepatic amebiasis. Dehydroemetine (available from the Centers for Disease Control and Prevention [CDC] has been successfully used but, because of its potential myocardial toxicity, is not preferred.

Broad-spectrum antibiotics may be added to treat bacterial super infection in cases of amoebic colitis and suspected perforation. Bacterial co-infection of amebic liver abscess has occasionally been observed (both before and as a complication of drainage), and adding

antibiotics to the treatment regimen is reasonable in the absence of a prompt response to nitroimidazole therapy (Stanley, 2003).

1.16 Metronidazole

Metronidazole is an antibiotic that is used to treat a wide variety of infections which marketed under the brand name Flagyl among others. It works by stopping the growth of certain bacteria and parasites (Brayfield, 2014). Metronidazole (1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole) is the drug of choice for the treatment of infections caused by anaerobic or microaerophilic microorganisms and has been used in clinical practice for >25 years. The molar mass of metronidazole is 171.15 g/mol. Metronidazole began to be commercially used in 1960 in France. It is on the World Health Organization's List of Essential Medicines, the most effective and safe medicines needed in a health system (WHO 2015). It is effective for giardiasis, trichomoniasis, and amebiasis. It is the drug of choice for a first episode of mild-to-moderate Clostridium difficile colitis. Metronidazole is available by mouth, as a cream, and intravenously (Cohen, 2010)

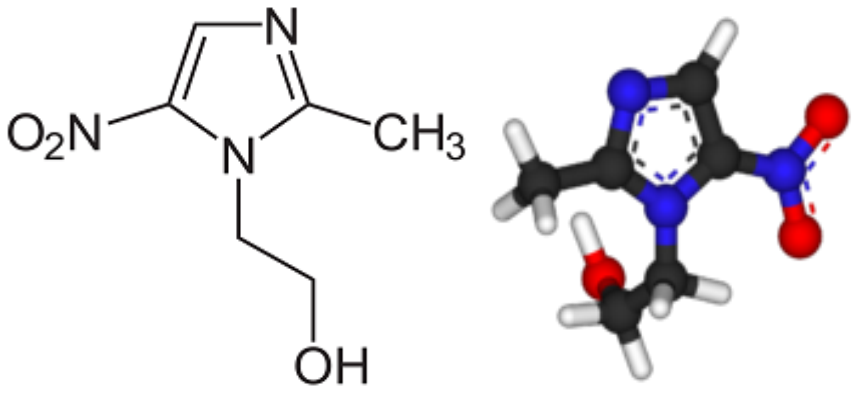

Figure 1.7: Chemical and 3D structure of Metronidazole

For the treatment of anaerobic infections and mixed infections, surgical prophylaxis requiring anaerobic coverage, Clostridium difficile-associated diarrhea and colitis, Helicobacter pylori infection and duodenal ulcer disease, bacterial vaginosis, Giardia lamblia gastro-enteritis, amebiasis caused by *E. histolytica* , acne rosacea (topical treatment), and Trichomonas infections (McEvoy, 2006).

1.17 Pharmacokinetics of Metronidazole

Metronidazole appears in cerebrospinal fluid, saliva, and breast milk in concentrations similar to those found in plasma. Less than 20% is protein bound. It is absorbed rapidly and completely if also given through orally. It is distributed very well into mucosa, saliva, bone and CSF. It is metabolized in liver. Routes of elimination are via urine (60% to 80%) and feces (6% to 15%). Renal Clearance is approximately 10 mL/min per 1.73 m^2. The plasma ½ life of metronidazole is about 8 hours (Thomson, 2006).

1.18 Metabolism of Metronidazole

Approximately 30-60% of an oral or IV dose of metronidazole is metabolized in the liver by hydroxylation, side-chain oxidation, and glucuronide conjugation. The major metabolite, 2-hydroxy metronidazole, has some antibacterial and antiprotozoal activity. Four other nitro-group-containing metabolites have been identified, each derived from side-chain oxidation of ethyl and/or methyl group. They include 1-acetic acid-2-methyl-5-nitroimidazole and 1-(2-hydroxyethyl)-2-carboxylic acid-5-nitroimidazole salt.

The liver is the main site of metabolism, and this accounts for over 50% of the systemic clearance of metronidazole. The 2 principal metabolites result from oxidation of side chains, a hydroxy derivative and an acid. The hydroxy metabolite

has a longer half-life (about 12 hr) and nearly 50% of the antitrichomonal activity of metronidazole. Formation of glucuronides also is observed. Small quantities of reduced metabolites, including ring-cleavage products, are formed by the gut flora. The urine of some patients may be reddish-brown owing to the presence of unidentified pigments derived from the drug (Hardman et al, 2001).

1.19 Mechanism of action of Metronidazole

Metronidazole is of the nitroimidazole class. It inhibits nucleic acid synthesis by disrupting the DNA of microbial cells (Eisenstein, 2007). Metronidazole can enter the cell through passive diffusion, where a nitro group is subsequently reduced to reactive cytotoxic nitro radicals by reduced ferredoxin or flavodoxin. Ferredoxin and flavodoxin function as electron acceptors of pyruvate. ferredoxin oxidoreductase, hydrogenase, and some other enzymes found specifically in microaerophilic bacteria and protozoan parasites. Under aerobic conditions, the nitro radicals can be oxidized by oxygen, which leads to futile cycling and detoxification of the drug. During this reaction, superoxide radical anions are formed that may also be toxic for the anaerobic organisms (Thomson, 2006).

Metronidazole is a prodrug. Unionized metronidazole is selective for anaerobic bacteria due to their ability to intracellularly reduced metronidazole to its active form. This reduced metronidazole then covalently binds to DNA, disrupt its helical structure, inhibiting bacterial nucleic acid synthesis and resulting in bacterial cell death. Treatment with Metronidazole is usually very effective (Brayfield, A, 2014).

1.20 Biological Half-Life of Metronidazole

The plasma half-life of metronidazole is reported to be 6-8 hours in adults with normal renal and hepatic function. The plasma half-life of metronidazole is not affected by changes

in renal function; however, the half-life may be prolonged in patients with impaired hepatic function. It has been observed from a study that half-life of metronidazole averaged 18.3 hours in adults with alcoholic liver disease and impaired hepatic function, (range: 10.3-29.5 hours) (McEvoy, 2006).

Literature review

2.1 In vitro Sensitivity of Different Brands of Antiamoebic Drugs (Metronidazole Tablets) Against Clinical Isolates of *Entamoeba histolytica* in Bangladesh

Entamoeba histolytica associated with high morbidity and mortality rate continues to be major public health problems throughout the world. The amoebic infection is primarily treated by instituting anti amoebic drug. The major drug of choice for treating amoebiasis is Metronidazole. The aim of the study was to evaluate in vitro sensitivity of different metronidazole tablets from Bangladeshi pharmaceuticals against clinical isolates of *E. histolytica*. This study was conducted with 12 different brands of Metronidazole tablets which collected from some big and small pharmaceuticals according to their business. The parasite count was adjusted to 3x106 parasites mL-1 in a medium. In vitro drug sensitivity assay of the samples was carried out by using 96 wells microtiter plates after treatment with different concentrations of metronidazoles. No statistical significance was observed in terms of viable parasites with the metronidazole tablets from three big pharmaceuticals at the concentration of 2.3, 3.5 and 4.6 μM when compared with the standard metronidazole. We conclude that brands from some big pharmaceuticals showed in vitro sensitivity against *E. histolytica* (Sarker et al., 2008).

2.2 *In vitro* activity of antiamoebic drugs against clinical isolates of *Entamoeba histolytica* and *Entamoeba dispar*

Amoebiasis is a major public health problem in tropical and subtropical countries. Although a number of antiamoebic agents are used for its treatment, yet the susceptibility data on clinical isolates of *Entamoeba histolytica* and *Entamoeba dispar* are not available. Therefore, the present study was aimed to assess the in vitro susceptibility of clinical isolates of *E. histolytica* and *E. dispar* to metronidazole, chloroquine, emetine and tinidazole. A total of 45 clinical isolates (15 *E. histolytica* and 30 *E. dispar*) were maintained in polyxenic cultures followed by monoxenic cultures. The results showed that all clinical isolates had a higher IC50 compared to reference strain to all the four drugs. *E. histolytica* isolates appeared to be more susceptible compared to *E. dispar* isolates and the reference

strain of *E. histolytica* to the metronidazole, chloroquine, emetine and tinidazole respectively. The results of the present study are in agreement with previous findings except that there was a significantly higher IC50 value of all four drugs to the clinical isolates as compared to the reference strain (Bansal et al., 2004).

2.3 Synthesis and antiamoebic activity of new 1-N-substituted thiocarbamoyl-3,5-diphenyl-2-pyrazoline derivatives and their Pd(II) complexes

The in vitro antiamoebic activity was evaluated against the HM1:IMSS strain of *Entamoeba histolytica* and the results were compared with the standard drug, metronidazole. All compounds have been characterized by means of elemental analyses, electronic, IR, 1H NMR and mass spectroscopic data, while the complexes have additionally been characterized by thermogravimetric patterns. The preliminary test results showed that the complexes had better antiamoebic activity than their respective ligands. Moreover, the complexes showed better inhibition of the test organism. The results suggest that the ligands and the complexes were found with IC50 lower than that of the standard drug metronidazole and thus are better inhibitor of growth of *E. histolytica* (Budakoti, 2006).

2.4 *In vitro* studies on the sensitivity of local *Entamoeba histolytica* to anti-amoebic drugs

The in vitro activity of drugs, namely dehydroemetine, ornidazole, metronidazole and tinidazole were determined against the locally isolated strains of *E. histolytica* in Thailand. The test was performed in liquid monophasic medium, i.e. liver marmite serum medium. In all, locally isolated strains from thirty hosts studied the minimal inhibitory concentration (MIC) for dehydroemetine ranged from 0.125 to 1 microgram/ml, ornidazole ranged from 0.0625 to 0.25 microgram/ml, metronidazole ranged from 0.0625 to 0.125 microgram/ml, and tinidazole ranged from 0.0625 microgram/ml to 0.25 microgram/ml. The MIC of dehydroemetine was significantly different from ornidazole, metronidazole and tinidazole. Metronidazole was superior to that of dehydroemetine but was not significantly different among ornidazole, metronidazole and tinidazole (Chintana et al., 1986).

2.5 Study of Combination Regimens of Anti-Amoebic Drugs for the Treatment of Amoebic Dysentery Caused by *E. histolytica*

Amoebiasis is one of the most common health problems in the developing countries. Poverty, ignorance, overcrowding, poor sanitation and malnutrition favor transmission and increase disease burden. The objective of the study is to determine the sensitivity of the combination regimens of anti-amoebic drugs against clinical isolates of *E. histolytica*. The clinical isolates of *E. histolytica* were treated with metronidazole, ornidazole, metronidazole+ornidazole, secnidazole, metronidazole + secnidazole, tinidazole, and metronidazole+tinidazole at different concentrations (12, 6, 3 &1.5 mg/ml). Result showed that combination of metronidazole and ornidazole (1.5 mg/ml) inhibit the growth of E histolytica and it has found significantly different when compared with the control ($p<0.05$). Combination of tinidazole and metronidazole at the concentration of 6 and 12mg/ml has also found statistically significant ($p<0.05$) to inhibit the growth of *E. histolytica* when compared with the control. We conclude that treatment with combination drugs may be a useful alternative to inhibit the growth of *E. histolytica* (Suki, 2015).

2.6 *Entamoeba bangladeshi* nov. sp., Bangladesh

Feces from both diarrheal and surveillance specimens were collected from a cohort of children living in Mirpur. A total of 2,039 fecal samples were examined microscopically (0.9% saline smear) and/or by fecal culture for amebic trophozoites and cysts. In 2010–2011, during analysis of feces, a new species was identified which was positive for *Entamoeba* organisms by microscopy or culture but negative for *E. histolytica, E. dispar,* and *E. moshkovskii* by PCR. This new spesies is named *Entamoeba bangladeshi* nov. sp. in recognition of the support of the Bangladesh community for this research. The incidence and effect of infection in infants by the newly recognized species *E. bangladeshi* await future epidemiologic studies (Royer et al., 2012).

2.7 *Entamoeba bangladeshi*: An insight

Molecular tools have the potential to differentiate microscopically similar gut micro-eukaryotes that may have significantly different relationships with the human host. Using broad range *Entamoeba* primers to amplify a section of the eukaryotic 18S small subunit ribosomal RNA gene a novel member of the *Entamoeba* family (*Entamoeba bangladeshi*) has recently been identified. Primers directed against a small subunit rRNA region conserved throughout the *Entamoeba* family were used to amplify a variable section of the gene, and its sequence confirmed that *E. bangladeshi* was a novel species that was most similar to the other members of the *Entamoeba* family, which infect humans, *E. histolytica* and *E. dispar*. The goal of this review is to place this species in the context of what is already known about this genus and to discuss the tools and data needed to elucidate the host-microbe relationship (Gilchrist et al., 2014).

Objective of the Study

3.1 Objective of the study

The objective of the study is to investigate the efficacy of Metronidazole drug against *E. histolytica* and *E. bangladeshi* at different concentrations.

Materials and Methods

4.1 Clinical isolation of cell

Clinical isolates of *E. histolytica* and *E. bangladeshi* were harvested from 24 hours old cultures and suspended in a LYI-S-2 medium. Axenic medium (LYI-S-2) consists of liver digest, yeast extract, iron, serum. The parasite count was adjusted to 1×10^6 parasites/ml in medium by haemocytometer (Mukhopadhyay, R.M, et al., 1996; Bansal, D. et al., 2004). Isolation is usually achieved by growing the species in an environment that was previously sterilized, and was thereby rid of contaminating organisms. This isolation was performed in the Parasitology Laboratory, Laboratory Sciences Division, International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b).

4.2 Sample collection

The drug metronidazole used in the study was procured as pure salt from SKF Pharmaceutical Industry, which is one of the leading companies of Bangladesh. The sample was properly checked for their physical appearance, stability, batch number, manufacturing date, expiry date, manufacturing license number, D.A.R. number. The parasites were collected from the research department of ICDDR'B in Mohakhali, Dhaka. This investigation was performed in the parasitology Laboratory of ICDDR'B during the time of September 2016 to February 2017.

4.3 Preparation of antimicrobial drug

1. 1.28 µg of metronidazole was weighted carefully by using a calibrated analytical balance

3. The calculated amount of sample was dissolved in 1ml distilled water.

4. The solution was mixed by shaking carefully.

5. Then the stock solution was stored in refrigerator.

4.4 Apparatus and Instruments

1) Eppendrof

2) Falcone tube

3) Micropipettes

4) Microtips

5) Beaker

6) Microtiter plate

7) Analytical balance

8) Vortex machine

9) Haemocytometer

10) Microscope

11) Incubator

12) Autoclave

13) Laminar flow

14) Trypan blue reagent

4.5 Incubator

An incubator is a device used to grow and maintain microbiological cultures or cell cultures. The incubator maintains optimal temperature, humidity and other conditions such as the carbon dioxide (CO2) and oxygen content of the atmosphere inside. Incubators are essential for a lot of experimental work in cell biology, microbiology and molecular biology and are used to culture both bacterial as well as eukaryotic cells.

Figure4.1: Incubator

4.6 Microscope

A **microscope** (from the Ancient Greek: "small" and "to look" or "see") is an instrument used to see objects that are too small to be seen by the naked eye. Microscopy is the science of investigating small objects and structures using such an instrument. Microscopic means invisible to the eye unless aided by a microscope.

Figure4.2: Microscope

4.7 Haemocytometer

The haemocytometer is a device usually used and originally designed to count blood cells or parasite cells. The haemocytometer was invented by Louis-Charles Malassez and consists of a thick glass microscope slide with a rectangular indentation that creates a chamber. This chamber is engraved with a laser-etched grid of perpendicular lines. The device is carefully crafted so that the area bounded by the lines is known, and the depth of the chamber is also known. It is therefore possible to count the number of cells or particles in a specific volume of fluid, and thereby calculate the concentration of cells in the fluid overall.

Figure 4.3: Haemocytometer

4.8 Autoclave

An autoclave is a pressure chamber used to carry out industrial processes requiring elevated temperature and pressure different from ambient air pressure. Autoclaves are used in medical applications to perform sterilization. Many autoclaves are used to sterilize equipment and supplies by subjecting them to high-pressure saturated steam at 121 °C (249 °F) for around 15–20 minutes depending on the size of the load and the contents. The autoclave was invented by Charles Chamberland in 1879.

Figure 4.4: Autoclave

4.9 Microtiter plate

A microtiter plate (spelled Microtiter is a registered trade name in the United States) or microplate or microwell plate or multiwell, is a flat plate with multiple "wells" used as small test tubes. The microplate has become a standard tool in analytical research and clinical diagnostic testing laboratories.

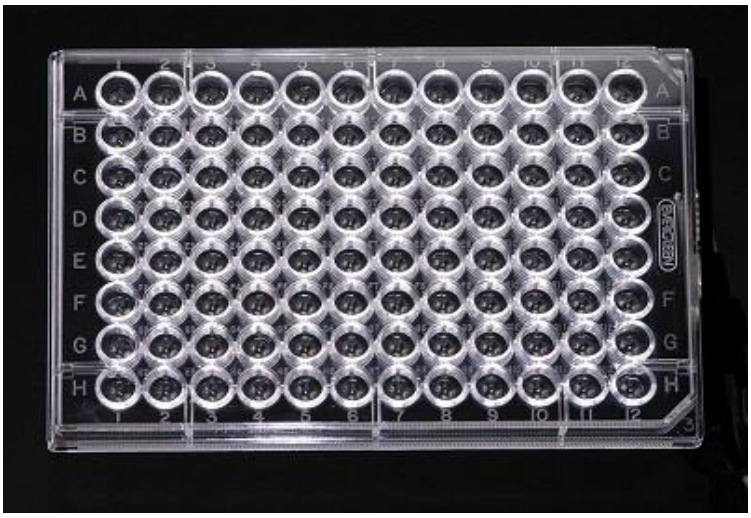

Figure 4.5: Microtiter plate

4.10 Vortex machine

A vortex mixer, or vortexer, is a simple device used commonly in laboratories to mix small vials of liquid. It consists of an electric motor with the drive shaft oriented vertically and attached to a cupped rubber piece mounted slightly off-center. As the motor runs the rubber piece oscillates rapidly in a circular motion. When a test tube or other appropriate container is pressed into the rubber cup (or touched to its edge) the motion is transmitted to the liquid inside and a vortex is created. Most vortex mixers are designed with 2 or 4-plate formats, have variable speed settings ranging from 100 to 3,200 rpm.

Figure 4.6: Vortex machine

Vortex mixers are quite commonplace in bioscience laboratories. In cell culture and microbiology laboratories they may be used to suspend cells. In a biochemical or analytical laboratory they may be used to mix the reagents of an assay or to mix an experimental sample and a dilutant.

4.11 Micropipette

Micropipettes are utilized in the laboratory to transfer small quantities of liquid, usually down to 0.1 uL. They are most commonly used in chemistry, biology, forensic, pharmaceutical, and drug discovery labs, among others.

Figure 4.7: Micropipette

4.12 In vitro drug sensitivity assay procedure

1. Drug sensitivity assay of the samples was carried out by using microtiter plates. In wells (A-1) to (F-1), 100 µl medium was added except (B-1) and (C-1)
2. Then in (C-1), 100 µl metronidazole stock solution was added and serial dilutions of the drugs were performed down {(C-1) to (F-1)}.

3. Then the plate was mixed properly.
4. After that 100 µl of the medium from the well (F-1) was discarded to maintain the equality of the concentration of the drugs.
5. Further 100 µl of parasite suspension (1×10^6 parasites/ml) was added to all the wells {(A-1) to (F-1)} except (B-1).
6. The final concentrations of the drugs were 1.28, 0.64, 0.32, 0.16, 0.08, 0.04 µg/ml.
7. Well (A-1) was control (contains only media and cells only).
8. Then plastic strip was used to cover the plate.
9. Plates were incubated at 37ºC for 4 hours.
10. After 4 hours the plate was taken from the incubator.
11. Then the viable parasites were counted by haemocytometer under microscope in each of the wells.

The entire procedure was carried out for both parasites *E. histolytica* and *E. bangladeshi.*

4.13 Statistical analysis

Data were analyzed with Prism 6 (GraphPad Software, La Jolla, CA, USA). One way analysis of Variance with a post hoc HolmeSidak multiple comparisons test was used for data analysis. Statistical significance was assigned to P values ($p=<0.05$).

Results

5.1 Determination of viable cell count of *E. histolytica* and *E. bangladeshi* after treatment with Metronidazole

The study was conducted with the active ingredient of metronidazole drug manufactured by SKF Pharmaceutical Ltd. The *in vitro* sensitivity of Metronidazole was carried out against clinical isolates of *E. histolytica* and *E. bangladeshi* by using different concentrations of the drug.

5.1.1 Mean viable cell count of *E. histolytica* and *E. bangladeshi* after 4 hours incubation of Metronidazole (1.28µg/ml)

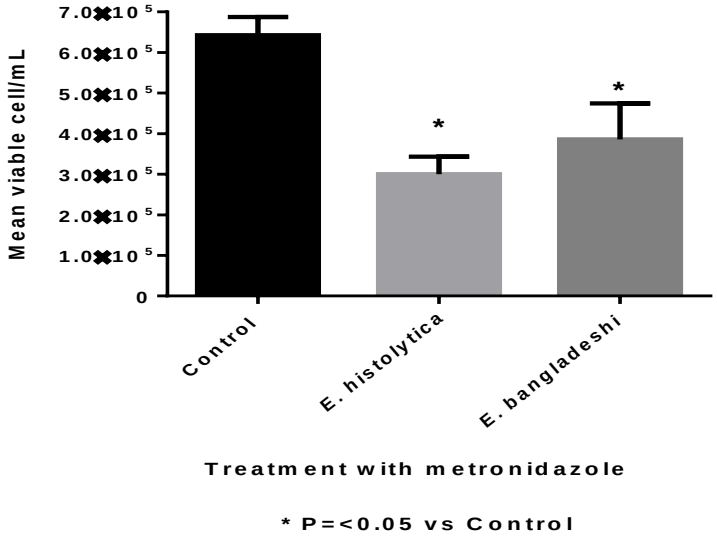

Figure 5.1: Mean viable cell counts of *E. histolytica* and *E. bangladeshi* after treatment with Metronidazole

Figure 5.1 shows the viable cell count of *E. histolytica* and *E. bangladeshi* at the concentration of 1.28 µg/ml after treatment with Metronidazole. The incubation period

was 4 hours at 37^0C. The mean viable count of *E. histolytica* and *E. bangladeshi* in control was $6.18X10^5$ parasite/ml and $6.61X10^5$ parasite/ml respectively. The mean viable cell count of *E. histolytica* was $3.0X10^5$parasites/ml and *E. bangladeshi* was $3.86X10^5$ parasites/ml at the concentration of 1.28 µg/ml after treating with Metronidazole. Significant differences have been observed between the control and treatments against *E. histolytica* and *E. bangladeshi* P=<0.05). However, the viable cell count of *E. histolytica* and *E. bangladeshi* were similar and no statistically significant difference has been observed between these two groups.

5.1.2 Mean viable cell count of *E. histolytica* and *E. bangladeshi* after 4 hours incubation of Metronidazole (0.64µg/ml)

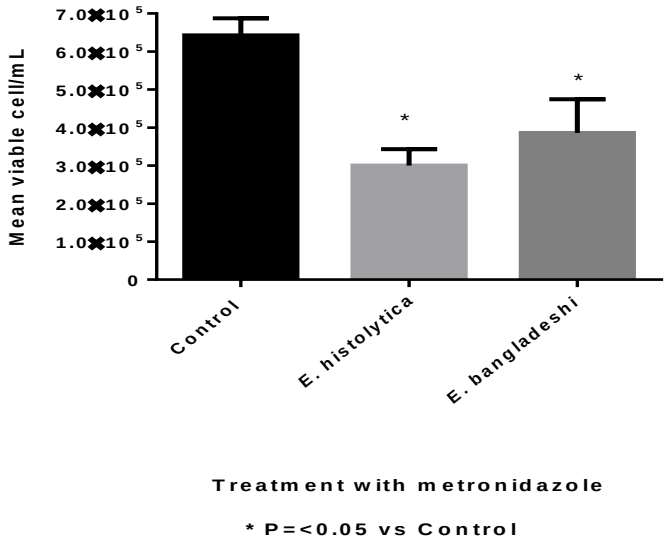

Figure 5.2: Mean viable cell counts of *E. histolytica* and *E. bangladeshi* after treatment with Metronidazole

Figure 5.2 shows the viable cell count of *E. histolytica* and *E. bangladeshi* at the concentration of 0.64µg/ml after treatment with Metronidazole. The cell was incubated for 4 hours at 37^0C with Metronidazole drug. The mean viable count of *E. histolytica* and *E.*

bangladeshi in control was 6.18X10^5 parasite/ml and 6.61X10^5 parasite/ml respectively. The mean viable cell count of *E. histolytica* was 3.32X10^5 and *E. bangladeshi* was 4.18X 10^5 at the concentration of 0.64 µg/ml after treating with Metronidazole. Viable cell count is significantly reduced in the treatment groups when compared with the control. However, the viable cell count of *E. bangladeshi* is similar when it was compared with cell count of *E. histolytica*.

5.1.3 Mean viable cell count of *E. histolytica* and *E. bangladeshi* after 4 hours incubation of Metronidazole (0.32 µg/ml)

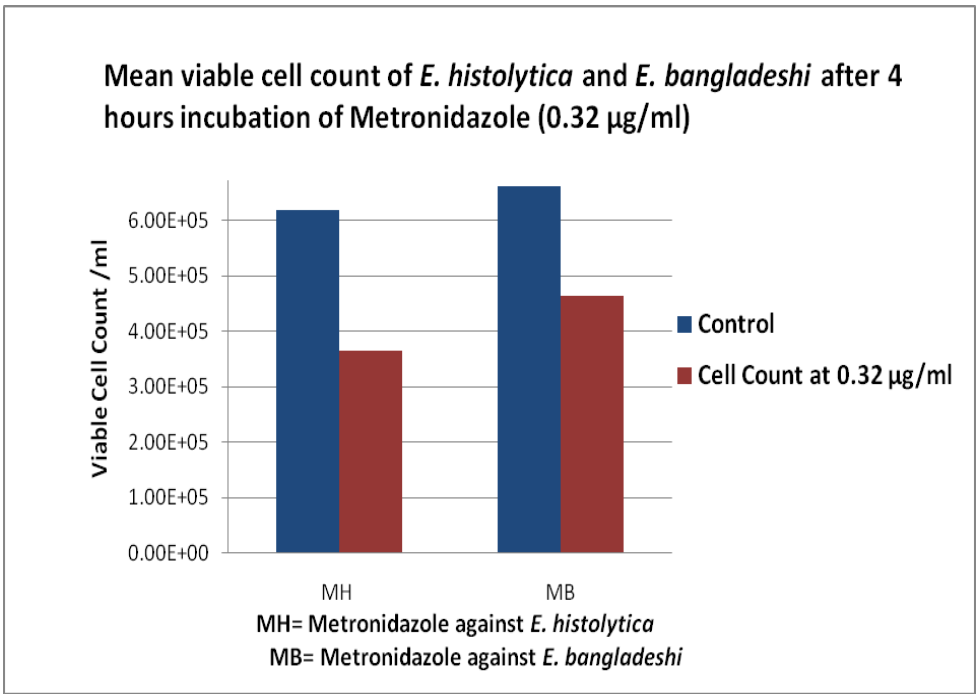

Figure 5.3: Mean viable cell counts of *E. histolytica* and *E. bangladeshi* after treatment with Metronidazole

Figure 5.3 shows the viable cell count of *E. histolytica* and *E. bangladeshi* at the concentration of 0.32 µg/ml after 4 hours incubation with Metronidazole. The incubation temperature was 37^{0}C. The mean viable count of *E. histolytica* and *E. bangladeshi* in control

was $6.18X10^5$ parasite/ml and $6.61X10^5$ parasite/ml respectively. The mean viable cell count of *E. histolytica* was $3.64X10^5$ parasites/ml and *E. bangladeshi* was $4.64X10^5$ parasites/ml at the concentration of 0.32 µg/ml after treating with Metronidazole. Significant differences have been observed between the control and treatments against *E. histolytica* and *E. bangladeshi* (P=<0.05). However, the viable cell count of *E. histolytica* and *E. bangladeshi* were similar and no significant difference has been observed between these two groups.

5.1.4 Mean viable cell count of *E. histolytica* and *E. bangladeshi* after 4 hours incubation of Metronidazole (0.16 µg/ml)

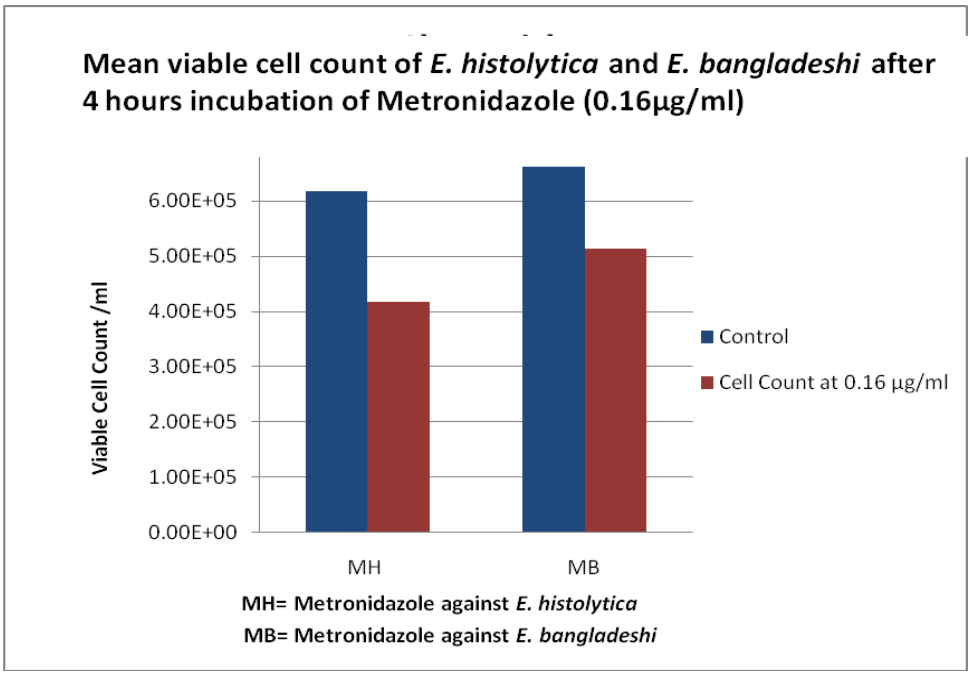

Figure 5.4: Mean viable cell counts of *E. histolytica* and *E. bangladeshi* after treatment with Metronidazole

Figure 5.4 shows the viable cell count of *E. histolytica* and *E. bangladeshi* at the concentration of 0.16 µg/ml after treated with Metronidazole. The incubation period was 4

hours at 37^{0}C. The mean viable count of *E. histolytica* and *E. bangladeshi* in control was 6.18X10^5 parasite/ml and 6.61X10^5 parasite/ml respectively. The mean viable cell count of *E. histolytica* was 4.18X10^5 parasites/ml and *E. bangladeshi* was 5.14X10^5 parasites/ml at the concentration of 0.16 µg/ml after treating with Metronidazole. Significant differences have been observed between the control and treatments against *E. histolytica* and *E. bangladeshi* (P=<0.05). However, the viable cell count of *E. histolytica* and *E. bangladeshi* were similar and no significant difference has been observed between these two groups.

5.1.5 Mean viable cell count of *E. histolytica* and *E. bangladeshi* after 4 hours incubation of Metronidazole (0.08 µg/ml)

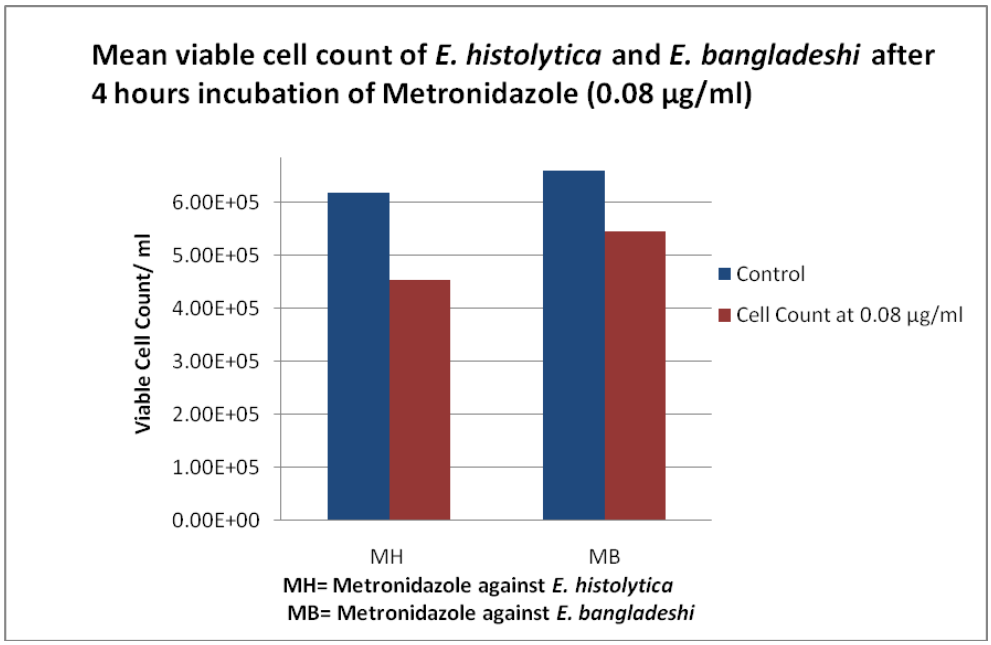

Figure 5.5: Mean viable cell counts of *E. histolytica* and *E. bangladeshi* after treatment with Metronidazole

Figure 5.5 shows the viable cell count of *E. histolytica* and *E. bangladeshi* at the concentration of 0.08 µg/ml after incubation with Metronidazole. The incubation period was 4 hours at 37^{0}C. The mean viable count of *E. histolytica* and *E. bangladeshi* in control

was 6.18X10^5 parasite/ml and 6.61X10^5 parasite/ml respectively. The mean viable cell count of *E. histolytica* was 4.54X10^5 parasites/ml and *E. bangladeshi* was 5.46X10^5 parasites/ml at the concentration of 0.08 µg/ml after treating with Metronidazole. Significant differences have been observed between the control and treatments against *E. histolytica* and *E. bangladeshi* (P=<0.05). However, the viable cell count of *E. histolytica* and *E. bangladeshi* were similar and no significant difference has been observed between these two groups.

5.1.6 Mean viable cell count of *E. histolytica* and *E. bangladeshi* after 4 hours incubation of Metronidazole (0.04 µg/ml)

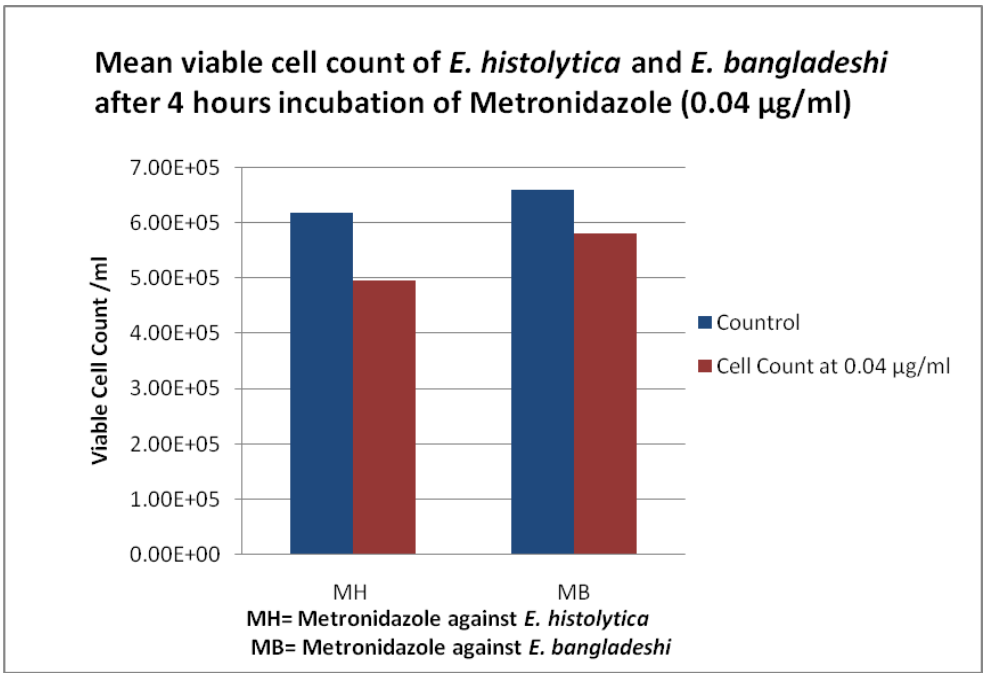

Figure 5.6: Mean viable cell counts of *E. histolytica* and *E. bangladeshi* after treatment with Metronidazole

Figure 5.6 shows the viable cell count of *E. histolytica* and *E. bangladeshi* at the concentration of 0.04 µg/ml after treatment with Metronidazole. The incubation period

was 4 hours at 37^0C. The mean viable count of *E. histolytica* and *E. bangladeshi* in control was $6.18X10^5$ parasite/ml and $6.61X10^5$ parasite/ml respectively. The mean viable cell count of *E. histolytica* was $4.96X10^5$ and *E. bangladeshi* was $5.82X\ 10^5$ at the concentration of 0.04 µg/ml after treating with Metronidazole. Viable cell count is significantly reduced in the treatment groups when compared with the control. However, the viable cell count of *E. bangladeshi* is similar when it was compared with cell count of *E. histolytica*.

5.2 *In vitro* sensitivity of different concentration of Metronidazole against *E. histolytica* and *E. bangladeshi* (based on viable cell count)

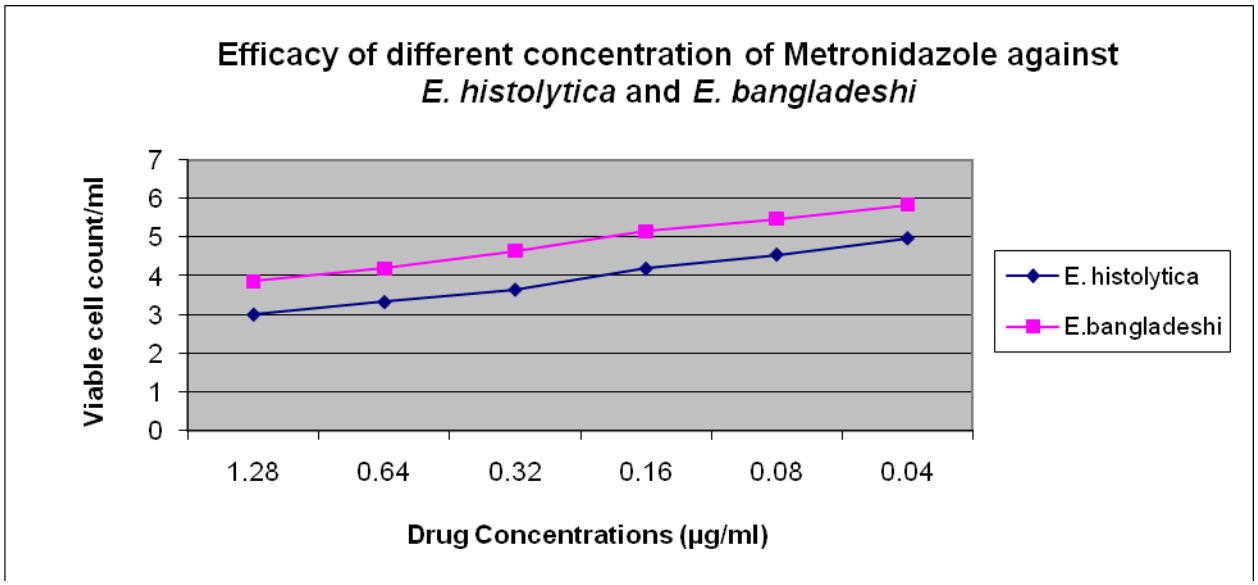

Figure 5.7: Efficacy of different concentration of Metronidazole against *E. histolytica* and *E. bangladeshi*

Figure 5.7 shows the viable cell count of *E. histolytica* and *E. bangladeshi* after treatment with different concentrations of Metronidazole after 4 hours incubation. The viable cell

count of *E. histolytica* is 3.0, 3.32, 3.64, 4.18, 4.54, 4.96 parasite/ml after treatment with 1.28, 0.64, 0.32, 0.16, 0.08, 0.04 µg/ml of metronidazole respectively. Similarly the viable cell count of *E. bangladeshi* is 3.86, 4.18, 4.64, 5.14, 5.46, 5.82 parasite/ml after treatment with 1.28, 0.64, 0.32, 0.16, 0.08, 0.04 µg/ml of metronidazole respectively. The figure shows that inhibition of parasite is occurred in a dose dependent manner. Cell inhibition is maximum at the highest concentration of Metronidazole.

Table 5.3 *In vitro* sensitivity of Metronidazole based on viable counts of *E. histolytica* after 4 hours incubation (n=7)

Drug concentration	MH (Mean±SD.)	MB (Mean±SD.)	P
Control	6.42 ±0.45	6.42 ±0.45	NS
1.28 µg/ml	3.0 ±0.43	3.86 ±0.89	NS
0.64 µg/ml	3.32 ±0.35	4.18 ±0.90	NS
0.32 µg/ml	3.64 ±0.40	4.64 ±0.91	NS
0.16 µg/ml	4.18 ±0.40	5.14 ±0.89	NS
0.08 µg/ml	4.54 ±0.47	5.46 ±0.85	NS
0.04 µg/ml	4.96 ±0.68	5.82 ±0.89	NS

Here, MH= Metronidazole against *E. histolytica*; MB= Metronidazole against *E. bangladeshi;* NS= No significant difference was observed.

All the data are compared with control.

Values are expressed as Mean±SD (n=7). *p<0.05 is used as level of significance. After 4 hours incubation, when the concentration of Metronidazole was 1.28 µg/ ml the viable count of *E. histolytica* was 3.0 parasite/ ml. When the concentrations were 0.64 µg/ml, the viable count of *E. histolytica* was 3.32 parasite/ ml. The viable count of *E. histolytica* was 3.64 parasite/ ml after treatment with 0.32µg/ml of metronidazole. When the concentrations of Metronidazole were 0.16µg/ml, 0.08µg/ml, 0.04µg/ml, the viable count of *E. histolytica* was 4.18, 4.54 and 4.96 parasite/ ml respectively. Similarly when the concentration of Metronidazole was 1.28 µg/ ml the viable count of *E. bangladeshi* was 3.86 parasite/ ml. When the concentrations were 0.64 µg/ml, the viable count of *E. bangladeshi* was 4.18 parasite/ ml. When the concentrations of Metronidazole were 0.32 µg/ml, 0.16µg/ml, 0.08µg/ml, 0.04µg/ml, the viable count of *E. histolytica* was 4.64, 5.14, 5.46 and 5.82 parasite/ ml respectively. Here no significant difference was observed.

5.4 *In vitro* sensitivity of different concentration of Metronidazole against *E. histolytica* and *E. bangladeshi* (based on non viable cell count)

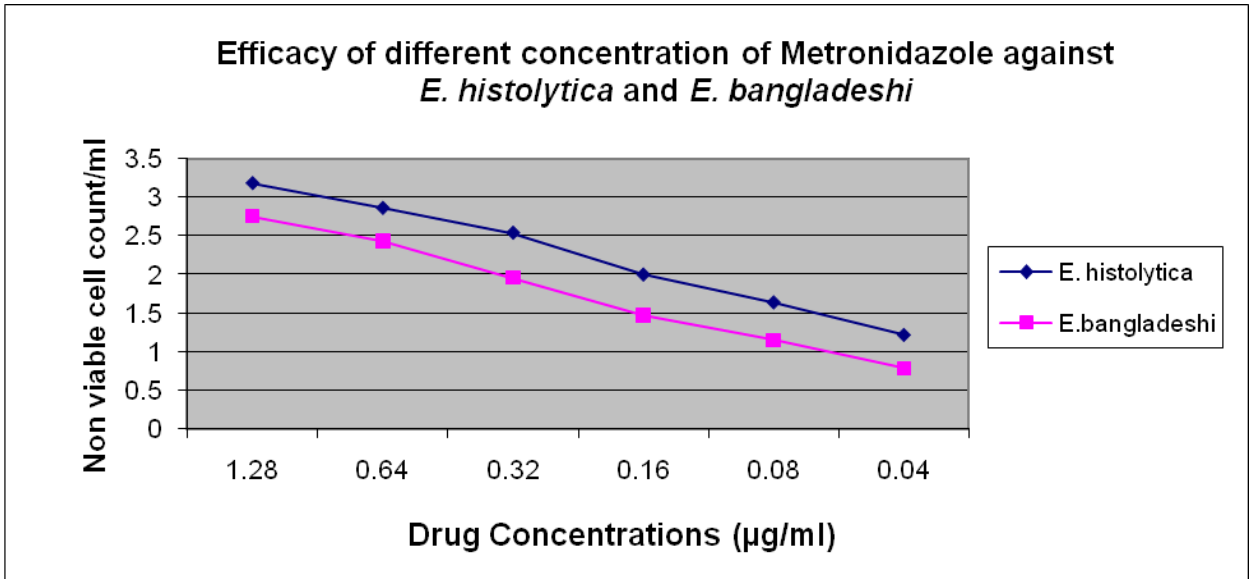

Figure 5.8: Efficacy of different concentration of Metronidazole against *E. histolytica* and *E. bangladeshi*

Figure 5.8 shows the non viable cell count of *E. histolytica* and *E. bangladeshi* after treatment with different concentrations of Metronidazole after 4 hours incubation. The non viable cell count of *E. histolytica* is 3.18, 2.86, 2.54, 2.0, 1.64, 1.22 parasite/ ml after treatment with 1.28, 0.64, 0.32, 0.16, 0.08, 0.04 µg/ml of metronidazole respectively. Similarly the non viable cell count of *E. bangladeshi* is 2.75, 2.43, 1.96, 1.47, 1.15, 0.79 parasite/ ml after treatment with 1.28, 0.64, 0.32, 0.16, 0.08, 0.04 µg/ml of metronidazole respectively. The figure shows that inhibition of parasite is occurred in a dose dependent manner. Cell inhibition is maximum at the highest concentration of Metronidazole.

5.5 Percentage of cell inhibition of two clinical isolates *E. histolytica* and *E. bangladeshi* by using IC_{50} calculation

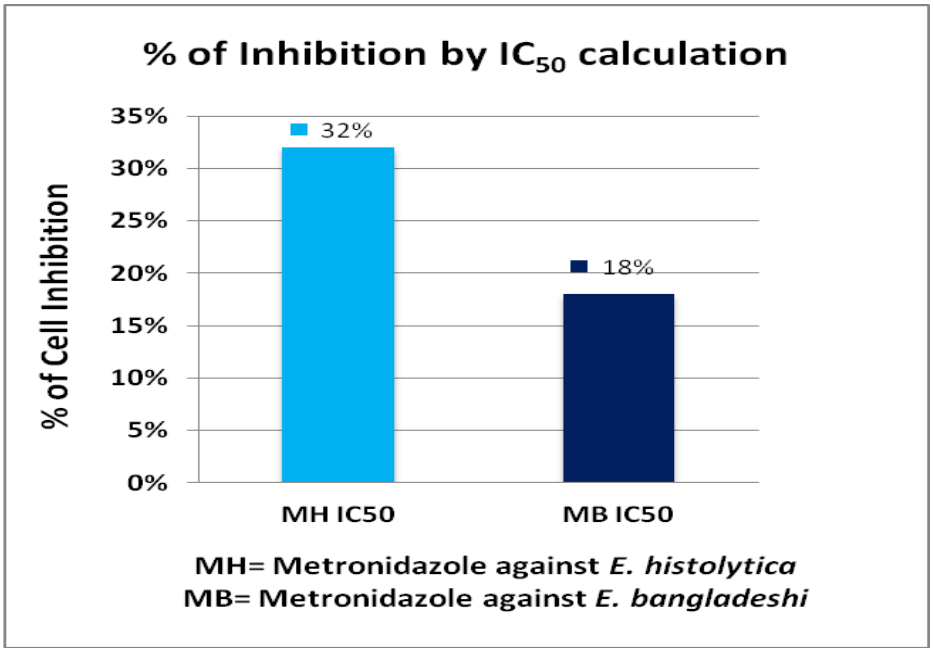

Figure 5.9: Percentage of cell inhibition after treatment with Metronidazole agaisnt *E. histolytica* and *E. bangladeshi*

Figure 5.9 shows that the IC_{50} values of metronidazole for the two clinical isolates *E. histolytica* and *E. bangladeshi*. The cell inhibition is 32% and 18% when Metronidazole treated against *E. histolytica* and *E. bangladeshi* respectively. That means the percentage cell inhibition of *E. bangladeshi* cells is lower than *E. histolytica* cells when they both are treated with different concentration of Metronidazole. The percentage of cell inhibition is calculated from an IC_{50} software named "Very Simple IC50 Tool Kit".

Discussion and Conclusion

Entamoeba histolytica produces amebic dysentery and liver abscess. Although amoebiasis (symptomatic infection with *E. histolytica*) is mostly prevalent in developing countries with inadequate sanitation, the presence of endemic foci in developed countries, the speed of modern travel, and the current prevalence of amebic infection in the homosexual community make the treatment of this infection a matter of concern for all physician. In 2010-2011 a new species was indentified from the feces of children which was named *Entamoeba bangladeshi* by the community of Bangladesh for this research (Royer et al., 2012).

In the present study clinical isolates of *E. histolytica* and *E. bangladeshi* were maintained by *in vitro* cultivation in axenic medium and were subjected to drug sensitivity tests against antiamoebic drugs Metronidazole. Several studies have been carried out to determine the sensitivity of Metronidazole against *E. histolytica*. However, no studies have been conducted to determine the sensitivity of Metronidazole against this new species *E. bangladeshi*. Our objective of the study was to determine the efficacy of anti-amoebic drug against clinical isolates of *E. bangladeshi*.

The clinical isolates of *Entamoeba histolytica* and *Entamoeba bangladeshi* were treated with Metronidazole at different concentrations. The concentrations of Metronidazole drug were 1.28µg/ml, 0.64µg/ml, 0.32µg/ml, 0.16µg/ml, 0.08µg/ml, and 0.04µg/ml in this present experiment. The number of viable counts is decreased when the concentrations of drugs are increased. The lowest number of viable count of both parasites was found when they were treated with 1.28 µg/ml of Metronidazole. The result shows that a significant differences have been observed between the control and treatments against *E. histolytica* and *E. bangladeshi* ($P=<0.05$). However, the viable cell count of *E. histolytica* and *E. bangladeshi* were similar and no significant difference has been observed between these two groups for all the concentrations. It also shows that the inhibition of parasite is occurred in a dose dependent manner which means cell inhibition is maximum at the highest concentration of Metronidazole for both parasites. After that the results also shows

that the percentage of cell inhibition of both parasites by calculating IC_{50}. Here the percentage of cell inhibition occurred 32% and 18% for *E. histolytica* and *E. bangladeshi* respectively. Therefore, the sensitivity of metronidazole for *E. bangladeshi* isolate is as efficient as for *E. histolytica*.

It has been shown from a study that the different metronidazole tablets from Bangladeshi pharmaceuticals were effective against clinical isolates of *E. histolytica.* These tablets were collected from different pharmaceuticals. The parasite count was adjusted to $3x10^6$ parasites mL-1 in a medium. No statistical significance was observed in terms of viable parasites with the metronidazole tablets when compared with the standard metronidazole (Sarker et al., 2008). In our study the parasite count was adjusted to $1x10^6$ parasites mL-1 in a medium. We used six different concentrations of metronidazole. The concentrations were 1.28, 0.64, 0.32, 0.16, 0.08, 0.04 μg/ml. From our study we have also found the sensitivity of metronidazole against *E. histolytica* after incubation. The incubation period was 4 hours.

Another study observed the sensitivity of the combination regimens of anti-amoebic drugs against clinical isolates of *E. histolytica*. The clinical isolates of *E. histolytica* were treated with metronidazole, ornidazole, metronidazole+ornidazole, secnidazole, metronidazole + secnidazole, tinidazole, and metronidazole+tinidazole tablets at different concentrations (12, 6, 3 &1.5 mg/ml). After 4 hours incubation the viable cells were counted by haemocytomer and the counts of *E. histolytica* for each concentration of drugs were compared to the control. In this study a significant difference had been between the combination and monotherapy of antiamoebic drugs (suki, 2015). In our study we used active ingredient of Metronidazole and carried out the same procedure to find out the sensitivity of the drug. Here we compared the viable cell counts of *E. histolytica* with the control and also observed significant differences between them.

In 2010–2011, during analysis of feces, a new species was identified which was positive for *Entamoeba* organisms by microscopy or culture but negative for *E. histolytica, E. dispar,* and *E. moshkovskii* by PCR. This new species is named *Entamoeba bangladeshi* nov. sp. in recognition of the support of the Bangladesh community for this research. The incidence and effect of infection in infants by the newly recognized species *E. bangladeshi* await future epidemiologic studies (Royer et al., 2012). As *E. bangladeshi* is a new novel species we do not know its pathogenicity. Moreover, no drug sensitivity studies have been carried out to determine the efficacy of the drug against this parasite. In our study we have determined the sensitivity of Metronidazole at different concentrations against *E. bangladeshi* and *E. histolytica*.

Bansal et al. reported the *in vitro* susceptibility of clinical isolates of *E. histolytica* and *E. dispar* to metronidazole, chloroquine, emetine and tinidazole. They maintained 45 clinical isolates (15 *E. histolytica* and 30 *E. dispar*). Results showed that all clinical isolates had a higher IC_{50} compared to reference strain to all the four drugs and *E. histolytica* isolates appeared to be more susceptible compared to *E. dispar* isolates (Bansal et al., 2004). In our study we also observed the IC_{50} value of metronidazole for both *E. histolytica* and *E. bangladeshi*. We have found that inhibition of parasite is occurred in a dose dependent manner and the percentage cell inhibition of *E. histolytica* is higher than *E. bangladeshi* cells when both are treated with different concentration of Metronidazole.

In conclusion the present study suggests that Metronidazole is also sensitive for *E. bangladeshi*. However, there is insufficient evidence to draw conclusions regarding the efficacy of Metronidazole drugs against *E. bangladeshi*. Further study is needed to draw a more evidence-based conclusion of effectiveness of antiamoebic drugs against the new parasite *Entamoeba bangladeshi.*

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