In vitro sensitivity of Ornil and Robic against clinical isolates of *Entamoeba Histolytica*

This thesis paper is submitted to the ***Department of Pharmacy, East West University*** *in conformity with the requirements for the Degree of* ***Bachelor of Pharmacy***

Prepared by
Mawarunnesa
ID # 2006-3-70-020

East West University
Department of Pharmacy

I would like to -
Dedicate this Research paper
To my beloved
Family members** and **Brothers
And to my respected supervisor -
Farhana Rizwan

DECLARATION

I, Mawarunnesa, hereby declare that the dissertation entitled "In vitro sensitivity study of Ornil and Robic against clinical isolates of *Entamoeba Histolytica* ", submitted by me to the Department of Pharmacy, East West University , in the partial fulfillment of the requirement for the award of the degree of Bachelor of Pharmacy (Honors) is a bonafide record of original research work carried out by me under the supervision and guidance of **Farhana Rizwan**, Senior Lecturer, Dept. of Pharmacy, East West University. The contents of this thesis, in full or in parts, have not been submitted to any other Institute or University for the award of any Degree or Diploma of Fellowship.

Mawarunnesa

Signature of the Candidate

Mawarunnesa

CERTIFICATE

This is to certify that, the research work on "*In vitro* sensitivity study of Ornil and Robic against Clinical isolates of *Entamoeba Histolytica*" submitted to the Department of Pharmacy, East West University, Mohakhali, Dhaka, in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy (B. Pharm) was carried out by Mawarunnesa (ID # 2006-3-70-020) under our guidance and supervision and that no part of the thesis has been submitted for any other degree. We further certify that all the resources of the information in thus connection are duly acknowledged.

Farhana Rizwan (*Sr. Lecturer*)
Research Supervisor
Department of Pharmacy
East West University
43, Mohakhali C/A
Dhaka- 1212

Sufia Islam Ph. D
Chairperson
Department of Pharmacy
East West University
43, Mohakhali C/A
Dhaka- 1212

ACKNOWLEDGEMENT

First and foremost I would like to thanks the Almighty Allah for providing me the gift of life, then; I would like to thanks my parents for their support, which helps me to complete my study.

I would like to place my great pleasure and sincere gratitude and respectful thanks to my Supervisor, **Farhana Rizwan**, Senior Lecturer, Department of Pharmacy, East West University, Bangladesh, for her adroit supervision, constructive suggestions, benevolent guidance and relentless encouragement at every stage of this research in throughout the period of the research work .It has been a great privilege for me to carry out this investigation under her dynamic guidance and supervision.

I would like to express my gratitude to the Chairperson, **Sufia Islam Ph. D.**, Department of Pharmacy, East West University, for giving me all the necessary support and facilities to complete this research work.

would like to express my sincere thanks to **Dr. Rashidul Haque,** Scientist and head of the Department of Parasitology laboratory and **Md. Abdullah Siddique** for their support in my study International Centre for Diarrheal Disease Research, Bangladesh (icddr,b).

ould like express thanks to **Mr. Ajoy Roy,** Laboratory Officer, Department of Pharmacy, East West University for his laboratory support in this research project.

uld like express thanks to my co-workers, and my friends for their unconditional help and support throughout the project.

List of Contents

		Page no.
List of Figures		**iii**
List of Tables		**v**
Abstract		**vi**
1.Introduction		**1-39**
1.1	Amebiasis	1
1.2	*Entamoeba histolytica*	2
1.3	*Entamoeba dispar*	5
1.4	Causes of Amebiasis	6
1.5	Symptoms of Amebiasis	7
1.6	Method of Diagnosis	14
1.7	Treatment of Amebiasis	20
1.8	Drugs Resistance in Amebiasis	25
1.9	Prevention of Amebiasis	26
1.10	Amebiasis in Bangladesh	28
1.11	Drugs Commonly Used in Amebiasis	29
Objective		**40**
Significance		**41**
Methods and Materials		**42-60**
4.1	Research Design	42
4.2	Collection of Clinical Isolation	42
4.3	Cultivation of *Entamoeba histolytica*	42
4.4	Media for Axenic Cultivation	44
4.5	Media for Xenic Cultivation	45
4.6	Preparation of Rice Starch	46
4.7	Diphasic Media	47
4.8	Monophasic Media	49
4.9	Axenic Culture Media	50
4.10	Establishment of Cultures	52
4.11	General Considerations for the Preparation of Xenic Culture	52
4.12	Elimination of Unwanted Organisms	53
4.13	Isolation	54
4.14	Axenization	55
4.15	Principles of Inducing Encystment of *E. histolytica*	57
4.16	Rexenization of Axenically Cultivated *E. histolytica*	58
4.17	Maintenance of Culture	59
4.18	Preparation of Antimicrobial Agent	59
4.19	In vitro Drug Sensitivity Assay	60

3.Results **61-70**

5.1 Measurement of Antiamebic Activity 61

5.1.1 Viable count (Standard and Ornil) 61

5.1.2 Non viable count (Standard and Ornil) 63

5.1.3 Viable count (Standard and Robic) 66

5.1.4 Non viable count (Standard and Robic) 68

4.Discussion **71-72**

5.Conclusion **73**

References **74-77**

List of Figures

Figure No.	Title of the Figure	Page no.
Figure. 1	Ameba	1
Figure. 2	*Entamoeba histolytica* in brain tissue	3
Figure. 3	Cyst stage of *E. histolytica*	3
Figure. 4	Life Cycle of *E. histolytica* including cysts and trophozoites stages	4
Figure. 5	Life Cycle of *E. histolytica*	5
Figure. 6	*Entamoeba dispar*	6
Figure. 7	Transmission of Amebiasis	7
Figure. 8	Algorithm of intestinal amebiasis	8
Figure. 9	Abdominal pain	9
Figure. 10	Bovine Viral Diarrhea Virus	10
Figure. 11	Amebic colitis	11
Figure. 12	*E. histolytica*- the causative agent of amebic dysentery and its cyst	11
Figure. 13	Acute appendicitis	12
Figure. 14	Acute bacterial peritonitis	12
Figure. 15	Amebic liver abscess	13
Figure. 16	Lung abscess	13
Figure. 17	Amebic brain abscess	13
Figure. 18	Characteristic features of trophozoites and cysts of intestinal ameba	16
Figure. 19	Different stages *of E. histolytica* inside the human body	23
Figure. 20	Mechanism of action of metronidazole	34
Figure. 21	Micropipette	43
Figure. 22	Microcentrifuge tube	43

Figure. 23	Autoclave	45
Figure. 24	Laminar air flow machine	45
Figure. 25	Incubator	46
Figure. 26	ELISA Plate	48
Figur . 27	Flow diagrams illustrating the stages in establishing luminal protists in culture	56
Figure. 28	Percentages of the viable counts *Entamoeba histolytica* after 24 hours incubation (Standard and Ornil)	63
Figure. 29	Percentages of the non viable counts *Entamoeba histolytica* after 24 hours incubation (Standard and Ornil)	65
Figure. 30	Percentages of the viable counts *Entamoeba histolytica* after 24 hours incubation (Standard and Robic)	67
Figure. 31	Percentages of the non viable counts *Entamoeba histolytica* after 24 hours incubation (Standard and Robic)	69

List of Tables

Table No.	Title of the Table	Page no.
Table. 1	Different drugs dosage regimens	22
Table. 2	The quantities composition of LYI-S-	52
Table. 3	Viable counts of *Entamoeba histolytica* after 24 hours incubation (Standard and Ornil)	62
Table. 4	Percentage of viable *Entamoeba histolytica* after 24 hours incubation (Standard and Ornil)	62
Table. 5	Non viable counts of *Entamoeba histolytica* after 24 hours incubation (Standard and Ornil)	64
Table. 6	Percentage of non viable *Entamoeba histolytica* after 24 hours incubation (Standard and Ornil)	64
Table. 7	Viable counts of *Entamoeba histolytica* after 24 hours incubation (Standard and Robic)	66
Table. 8	Percentage of viable *Entamoeba histolytica* after 24 hours incubation (Standard and Robic)	67
Table. 9	Non viable counts of *Entamoeba histolytica* after 24 hours incubation (Standard and Robic)	68
Table. 10	Percentage of non viable *Entamoeba histolytica* after 24 hours incubation (Standard and Robic)	69

ABSTRACT

Two brands of Ornidazole, Ornil and Robic are 5-nitroimidazole derivative drug which has antimicrobial action. These drugs are used in the treatment of protozoal infections and also in the treatment and prophylaxis of anaerobic bacterial infections. The clinical isolates of *Entamoeba histolytica* were treated with two brands of Ornidazole, Ornil and Robic at different concentration. The experimental concentrations are 0.14, 0.29, 0.58, 1.15, 2.3, 4.6 and 9.2 μM. A control group was made to measure the change in the viable counts and was put into the ELISA plate. Each ELISA plate contained different concentration of Ornil and Robic 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 which demonstrated that Ornil and Robic having good sensitivity against clinical isolates of *Entamoeba histolytica.*

Keywords: Ornil, Robic, Antimicrobial, *Entamoeba histolytica*, ELISA, Viable and Non-viable, Sensitivity.

Introduction

1. Introduction

Amebas are unicellular organisms common in the environment: many are parasites of vertebrates and invertebrates. Parasitology is the study of parasites and as such does not include bacterial, fungal or viral parasites. Human parasites are separated into intestinal and blood-borne parasites. For a parasite to be defined as intestinal it must have an intestinal life-cycle stage, though it may have life-cycle stages in the heart, circulation, lung, tissue, other animals or the environment. Parasites found in the intestines can be categorized into two groups: Protozoa and Helminthes. Protozoa are single celled organisms. There are four classes of Protozoa commonly found in concentrated fecal samples. These are differentiated by the method of motility. Protozoa include

- ✓ *Entamoeba*, *Giardia*, *Trichomonas*,
- ✓ *Cryptosporidium*,
- ✓ *Isospora*, *Pneumocystis* and
- ✓ *Balantidium*. [7]

1.1 Amebiasis

Amebiasis is an infection caused by pathogenic amebas or protozoan, especially *Entamoeba histolytica*. It is also called as amebiasis, amebism and amebic dysentery. Amebas get their name from the Greek word *amoibe* (meaning "change"), because their shapes are constantly changing. An ameba cell is composed of protoplasm differentiated into a cytoplasmic membrane, cytoplasm and a nucleus. The cytoplasm contains granules, as well as vacuoles filled with food, wastes, water, and possibly gases.

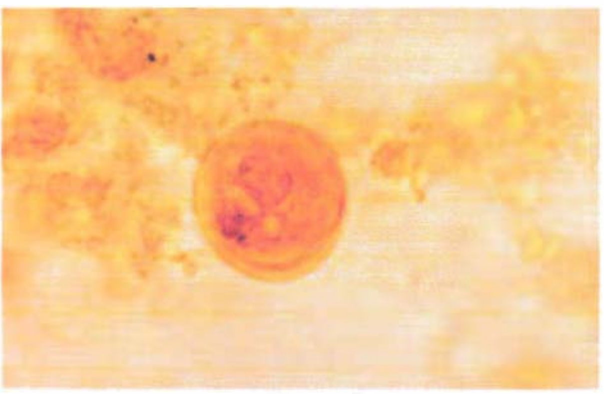


Fig. 1: ***Ameba***
http://www.mentalfloss.com/blogs/archives/10643

...ytoplasmic membrane is selective, permitting the passage of certain soluble nutrients into ... and waste material out of the cell. These microorganisms are almost constantly in ... They move by sending out portions of their bodies in one direction so that the whole cell ... into the location of the projection, called the pseudopodium. Reproduction in amebas ... binary fission. [26] Microorganisms obtain their food by pseudopodia that extend ... pores in the shells. [38]

... *Entamoeba histolytica*

... intestine get inflamed in this condition because of parasitic microorganism action, ...*moeba histolytica*. *Entamoeba histolytica* was first described by Fedor Lo¨sch in 1875 ...sburg, Russia. The genus *Entamoeba* contains many species, six of which

...moeba histolytica,

...oeba dispar,

...moeba moshkovskii,

...moeba polecki,

...ba coli and

...ba hartmanni

...g in the human intestinal lumen. *Entamoeba histolytica* is the only species ...ated with pathological sequelae in humans; the others are considered ... About 90% of people infected with *E. histolytica* have no symptoms of disease ... clear their infection, while the remaining 10% develop invasive disease. ... of untreated individuals with asymptomatic infection coming from areas ...asis develop symptoms of invasive amoebic disease within one year. ...ica also causes liver abscess, respiratory tract infections, and cerebral and ...sis. [21]

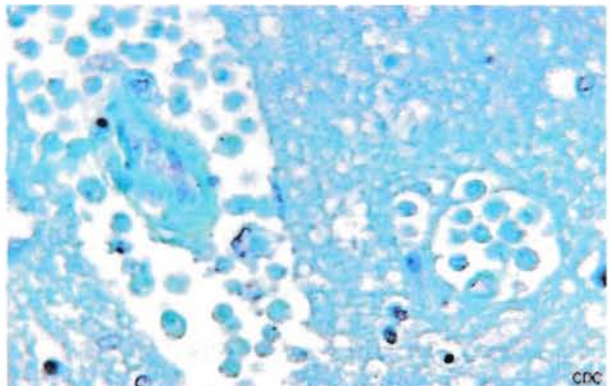


Fig. 2: *Entamoeba histolytica* in brain tissue
http://aapredbook.aappublications.org/week/

Life Cycle

Human is the main host of the *Entamoeba histolytica*. Usually source of the infection is also the human body. It does not require any intermediate host in the life cycle. *E. histolytica* has two principle stages in their life cycle.

Cyst stage

For protection during periods unfavorable for normal growth, some amebas can from cysts. The cyst often undergoes nuclear multiplication without cell division, resulting in several nuclei within single cysts. [24] Mature cyst of *E. histolytica* is spherical in shape and the diameter is 12 – 15 μm. Generally the infection occurs at the cyst stage.

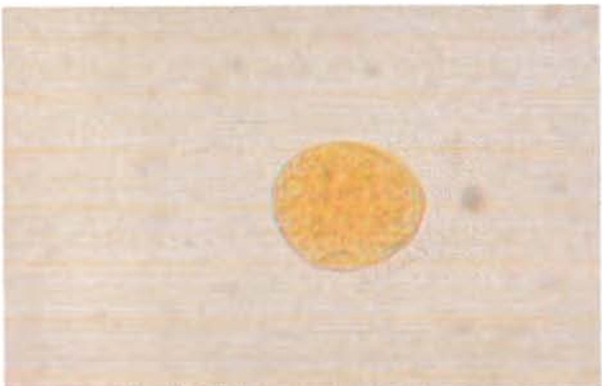
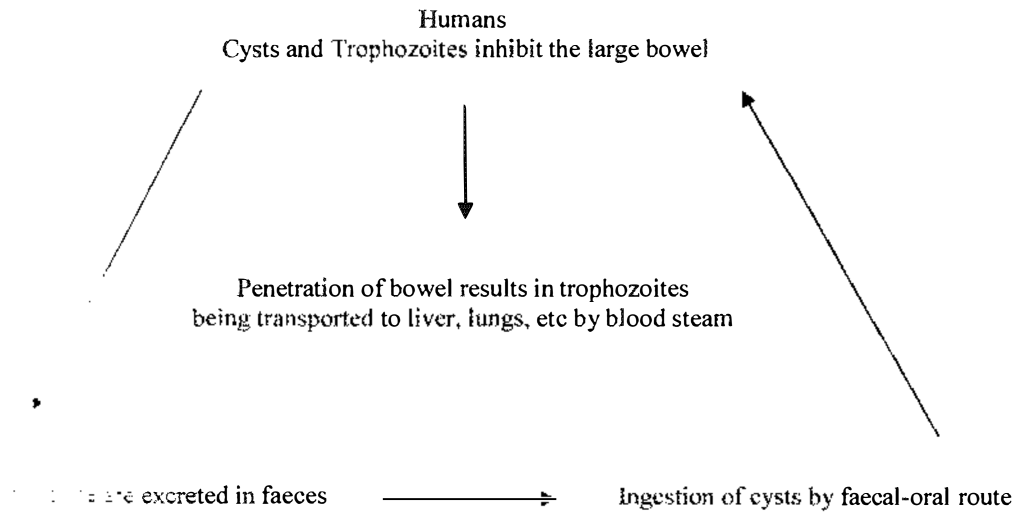


Fig. 3: Cyst stage of *E. histolytica*
(Source: Atlas of Medical Parasitology)

stage is immediately infective and the cysts pass through the feces. Cysts cause the mination of food and water; it will further produce the infections of the people who take ntaminated food, water or hand. If the cysts survive the acidic stomach, they transform to trophozoites in the small intestine. Excystment occurs at the lower part of the small . The immature amoebas move to the large intestine and it is the main site of the . [28]

zoite stage

mans *E. histolytica* lives and multiplies as a trophozoite. Trophozoites are oblong and -20 μm in length. In order to infect other humans they encyst and exit the body. tes migrate to the large intestine where they live and multiply by binary fission. If es are ingested, they are killed by the gastric acid of the stomach. [18] Occasionally es might be transmitted during sexual intercourse. Both cysts and trophozoites are present in the feces. Cysts are usually found in firm stool, whereas trophozoites are e stool. Only cysts can survive longer periods and infect other humans. [28]



Cycle of ***Entamoeba histolytica*** **including cysts and trophozoites stages**
http://www.soton.ac.uk/~ceb/Diagnosis/Vol1.htm

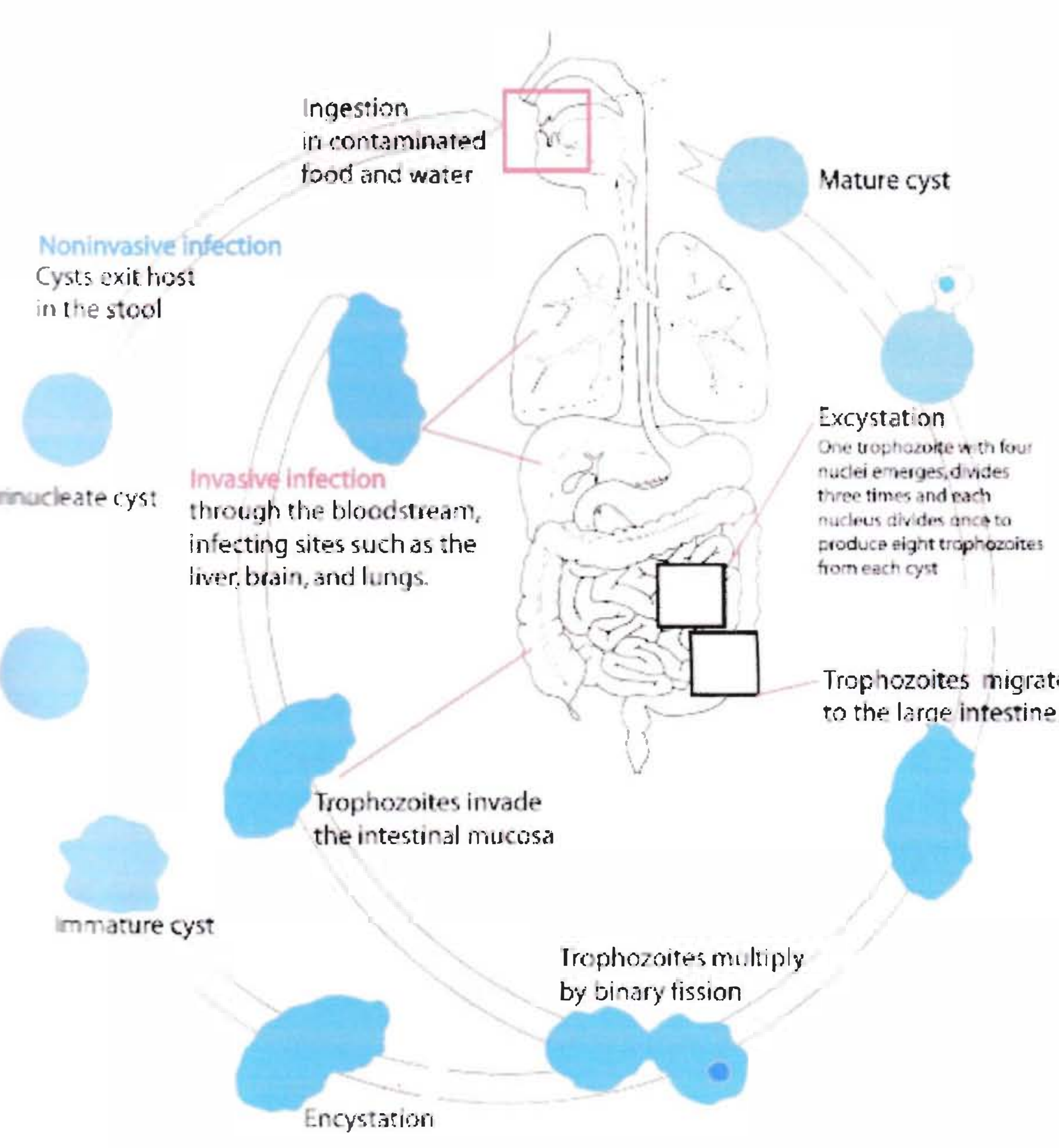


Fig. 5: Life cycle of *E. histolytica*
http://www.wikidoc.org/index.php/Entamoeba_histolytica

Entamoeba dispar

This is another form of amebiasis, and is caused by *E.dispar*. It is very mild and mostly asymptomatic. Many times, patients misdiagnosed to have *E. histolytica* infection, but who have no symptoms whatsoever, are actually infected with *E. dispar.* [12]

In 1925, Brumpt formulated the theory that the difference between many asymptomatic amebic infections and those of individuals with amebic disease could be correlated with the existence of two distinct but morphologically identical species, namely, *E. histolytica* (which is capable of causing invasive disease) and *E. dispar* (which never causes disease). In 1993, after 68 years the original discovery of *E. dispar,* these two were formally accepted as different yet closely related species on the basis of extensive genetic, immunological, and biochemical analyses. [21]

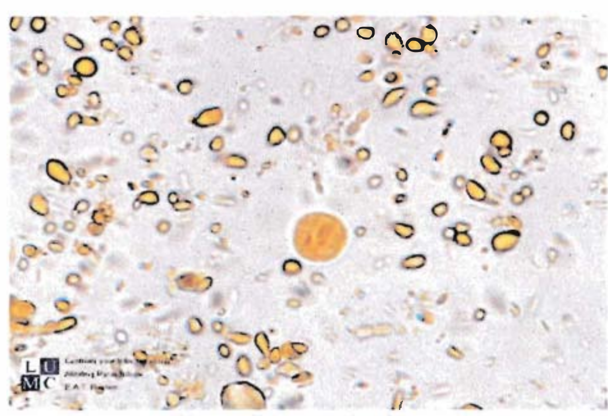


Fig. 6: *Entamoeba dispar*
http://www.parasitologie.nl/index

1.4 Causes of Amebiasis

Amebiasis is most prevalent in tropical and subtropical countries where standards of public hygiene and sanitation may be low. About 5,000 to 10,000 cases are diagnosed each year in the U.S. leading to about 20 deaths annually. It is most serious in infants, the elderly and debilitated people. Some possible causes of amebiasis include:

- Eating or drinking contaminated water or food is one of the primary causes of amebiasis
- Even vegetables grown in soil contaminated by feces can transmit the disease
- It may be transmitted from one person to other through direct contact
- Eating or swallowing anything that has touched the stool of a person who is infected with *Entamoeba histolytica.*
- Touching and bringing to the mouth, cysts (eggs) picked up from surfaces that are contaminated with *Entamoeba histolytica.*
- Eating a food on which mosquito had sat, after sitting on the stool of a person infected with *Entamoeba histolytica*, may lead to amebiasis

- Eating Non- vegetables foods (meat and intestines of animals - goat, pig, beef, etc.), may lead to the condition of amebiasis
- Unhygienic Conditions and Poor Sanitation areas are more susceptible to amebiasis
- Amebic dysentery can also be spread by anal sex. [11]

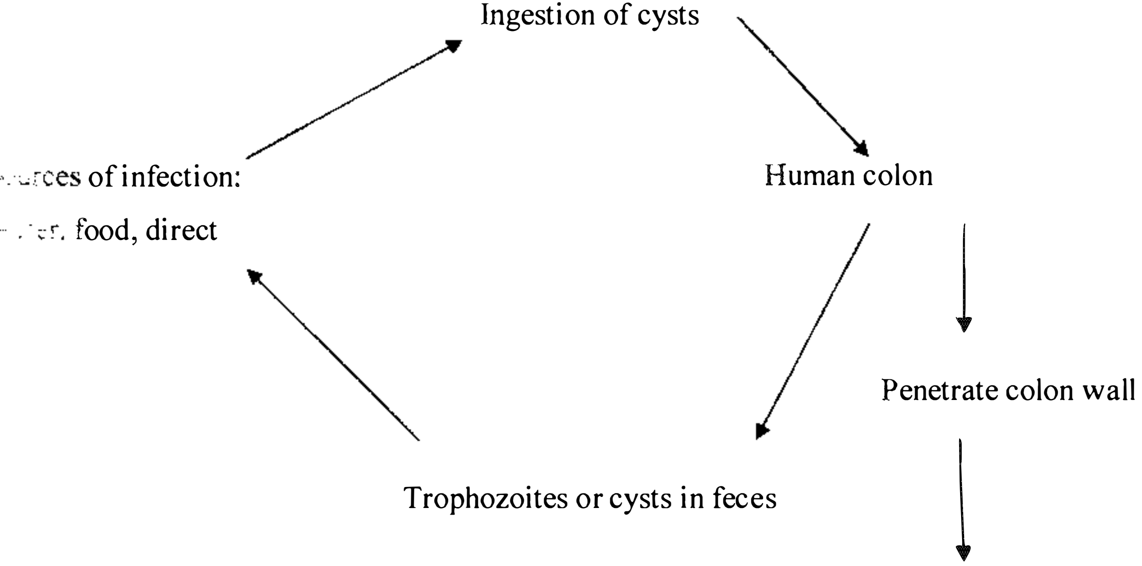


Fig. 7: Transmission of Amebiasis
http://www.bing.com/images/searchq=amebiasis

...ptoms of Amebiasis

... be occurred in two ways. One is Intestinal amebiasis and the other is extra- ...asis.

...biasis

... survives in the big bowel without causing any symptoms, but sometimes ... lining of the big bowel, causing intestinal disease called intestinal amebiasis. ... type of amebic infection is asymptomatic cyst passage. Symptomatic patients ... abdominal pain and diarrhea and later develop dysentery. Fulminant

ction with high grade fever, severe abdominal pain and profuse diarrhea occurs in children in patients receiving steroids. Severe gastric distention of the bowel can occur. Amebomas mmatory mass lesion developing in chronic amebiasis) can present like a malignancy. [30]

ry

Sub acute onset, bold/ mucous in stool, travel/residence in endemic area

l sign and symptoms

⇩

Diarrhea/dysentery, abdominal pain, weight loss, fever (>38°C) in <20%

⇩

tests to **separate invasive** **ry diarrhea**

Lactoferrin, fecal leucocytes, occult blood

⇩

***E. hisolytica* diagnostic tests**

⇩

on of stool *E.hisolytica* Ag- detection test or stool PCR plus Serology

Fig. 8: Algorithm of intestinal amebiasis
http://cmr.asm.org/cgi/content/full/16/4/713

ebiasis

s of fever and right upper abdominal pain. Jaundice is rare. Amoebic present as pyrexia of unknown origin. The abscess can sometimes peritoneal or pericardial cavities. The main symptoms of amebiasis are

Abdominal pain: Abdominal pain is any type of pain or discomfort that occurs in any area from the lower chest to the groin. Abdominal pain is symptom of a wide variety of mild to serious diseases, disorders and conditions. Abdominal pain can result from infection, malignancy, inflammation, trauma, obstruction and other abnormal processes. [41]

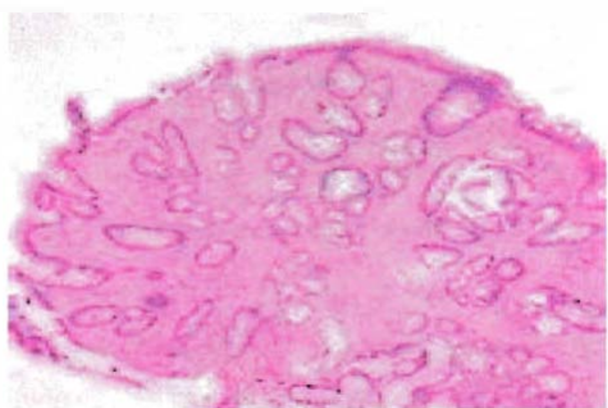


Fig. 9: Abdominal pain
http://pathology2.jhu.edu/gicases/case.cfm?case=11

Rectal pain: Rectal pain is the symptom of pain in the area of the rectum. A number of different causes have been documented. Differential diagnosis Anal fissures are the most common cause of rectal pain. They involve a tear in the anal canal probably due to trauma from defecation and are usually treated effectively with sitz baths, stool softeners, and analgesics. [41]

Diarrhea: Diarrheal illness is a significant worldwide cause of morbidity and mortality. Those resulting in gastrointestinal illness are greatest in developing countries due to the various routes of transmission specifically tied to hygiene. Organisms commonly causing diarrheal illness are *Entamoeba histolytica, Cyclospora, Giardia sp* etc. Diarrheal illness can be classified as acute (14 days in duration), persistent (14 days) and chronic (30 days). [26]

The figure below is Bovine viral diarrhea virus (BVDV). Coloured transmission electron micrograph of a cultured bovine embryonic testes cell infected with the BVDV virus, a type of pestivirus. The viruses are the small, red particles inside circular enclosures of rough endoplasmic reticulum (blue). The viruses are also seen in tubular structures, and some of these viruses show distinct envelopes. The red regions bound to the endoplasmic reticulum (blue seen in cross section) are ribosomes.

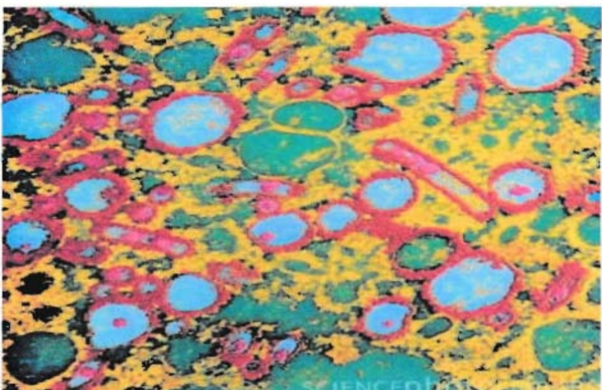


Fig. 10: Bovine Viral Diarrhea Virus
http://www.sciencephoto.com/images/

Fever : A fever usually means the body has raised its temperature to fight an infection or condition. The most common causes are infectious viruses such as cold or flu. Also possibilities include gastroenteritis, hepatitis, sinusitis, tonsillitis, otitis media, urinary tract infections, measles, roseola, prostratitis, mononucleosis, dental abscess, tuberculosis, and drug reactions. The most common causes of fever in children include cold, flu, otitis media, throat infection, UTI, or roseola, but there are numerous other possibilities. [41]

Bloody stool : Blood in the stool is an abnormal, potentially critical condition, in which there is blood mixed in with the bowel movement or feces. Blood in the stool is also called bloody stool or melena and is often a sign of gastrointestinal bleeding. Blood in the stool is sometimes accompanied by frank bleeding from the rectum or rectal bleeding. [41]

Amoebic Dysentery : Ingestion of water or food containing cysts of the protozoan *Entamoeba histolytica* may lead to a severe and sometimes fetal dysentery syndrome. The ingested cysts pass through the stomach; the trophozoites emerge in the lower small intestine and multiply in the lumen of the colon. Cysts reform and are shed in feces. [36]

Amoebic Colitis: Colitis amebiasis is usually characterized by bloody and mucous diarrhea, abdominal pain and anal discomfort. However, there is unusual manifestation of colitis amoebic, such as occasional dripped anal bleeding, which sometimes spouted. An acute or

chronic inflammation of the colon can be caused by viruses (e.g. cytomegalovirus), bacteria protozoa (e.g. ameba) or immunologic factors (inflammatory bowel disease which includes Crohn's colitis and ulcerative colitis). [41]

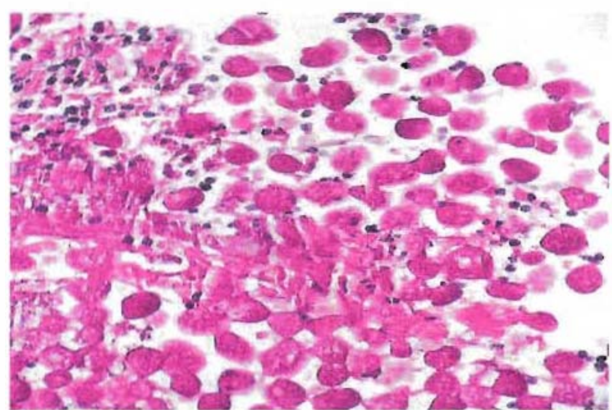


Fig. 11: Amebic colitis
http://www.bing.com/images/ q=amebiasis&view

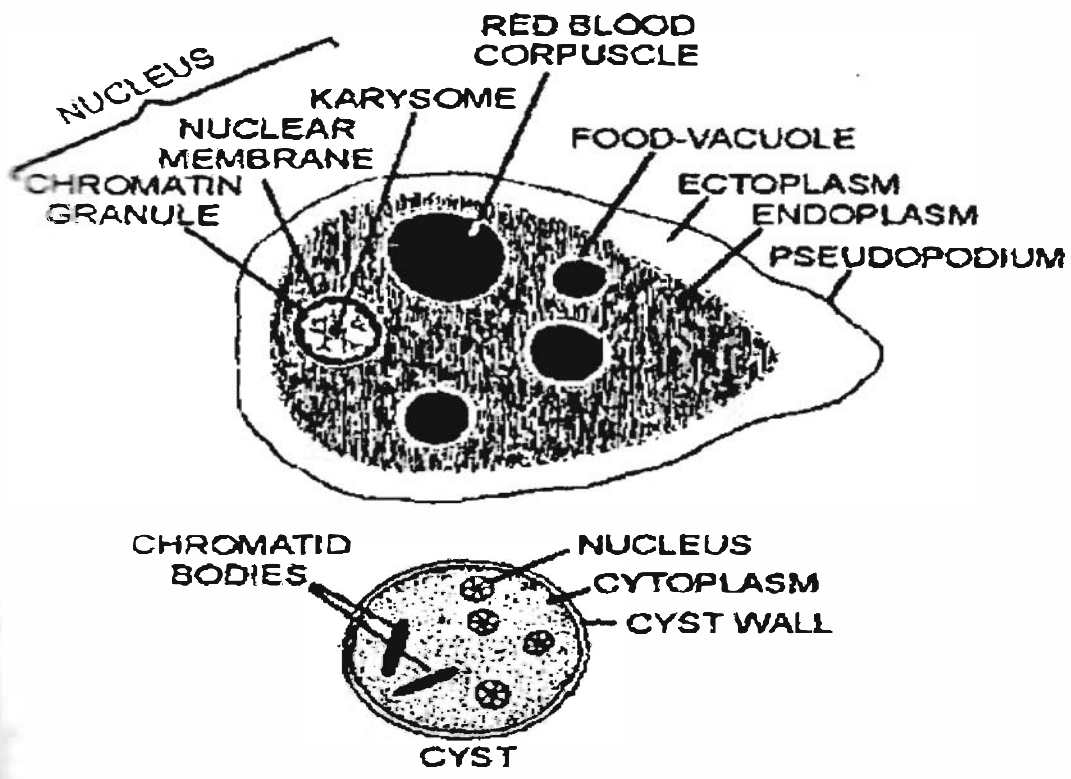


Fig. 12: ***Entamoeba histolytica-*** **the causative agent of amebic dysentery and its cyst**
http://www.tutornext.com/amoebic-dysentry-ascarlasis-filariasis-ringworms/

Appendicitis: Acute appendicitis is a rapidly progressing inflammation of a small part of the large intestine called the appendix. Acute appendicitis is a medical emergency that generally requires prompt removal of the appendix to prevent life-threatening complications, such as ruptured appendix and peritonitis. In contrast, chronic appendicitis develops slowly, has milder symptoms, and can often be treated with antibiotics. The appendix is a pouch-like structure located in the lower right quadrant of the abdomen near the area where the small intestine links into the large intestine. The exact function of the appendix is not known, although it might be useful in protecting beneficial bacteria of the colon. [41]

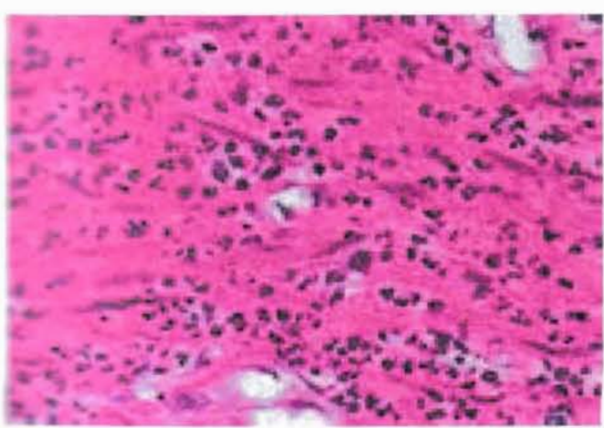


Fig. 13: Acute appendicitis
http://oac.med.jhmi.edu/pathconcepts/showimage.cfm?tutorialid

Fig. 14: Acute bacterial peritonitis
http://www.health-res.com/sub-acute-bacterial-peritonitis/

Peritonitis: Peritonitis is a serious disorder caused by an inflammation of the peritoneum, most often due to a bacterial infection. The peritoneum is a two-layered membrane that lines the abdominal cavity and encloses the stomach, intestines, and other abdominal organs. The inflammation may affect the entire peritoneum, or be confined to a walled-off, pus-filled cavity. A rupture anywhere along the gastrointestinal tract is the most common pathway for entry of an infectious agent into the peritoneum. [41]

Liver abscess: Amebic liver abscess is the most common extraintestinal manifestation of amebiasis. Amebic liver abscess is rarely seen among children. The majority of patients are

males between 20 and 40 years of age. Amebic liver abscess might be mistakenly diagnosed as pyogenic abscess, necrotic hepatoma or echinococcal cyst. [34]

Lung abscess: Lung abscess is known as pulmonary amebiasis, the second most common extra-intestinal pattern of infection which is frequently associated with amebic liver abscess. It occurs in 2-3% of patients with invasive amebiasis. The formation of a localized collection of puss in a cavity in the lungs is called lung abscess. Abscesses can form in almost any part of the body. [48]

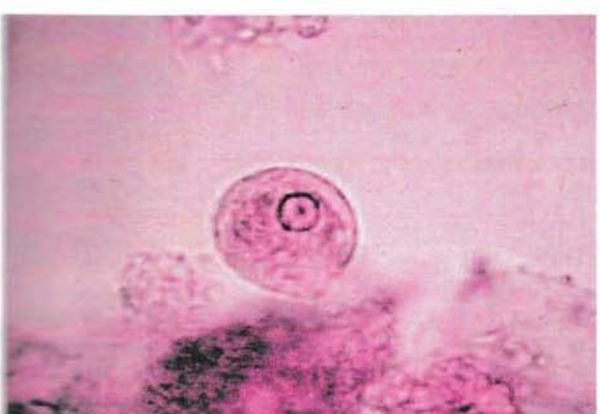


Fig. 15: Amebic liver abscess
http://www.health-pic.com/entamoeba-histolytica-incubation

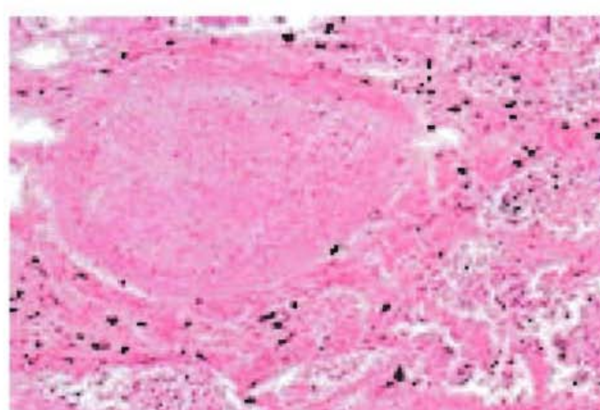


Fig. 16: Lung abscess
http://www.microscopyu.com/pathology/lungabscess

Amebic abscess of the brain: Most lesions of the brain are single and occur in the cortex of the cerebrum. There is an area of liquefaction of neuronal and glial cells with a mild inflammatory reaction but a limiting capsule rarely is present. Trophozoites may be found in viable tissue in the wall of the abscess or in the thick, yellowish or brownish pus. Fever, headache, vomiting and disturbances of vision and memory usually suggest meningitis or a space occupying lesion. [32]

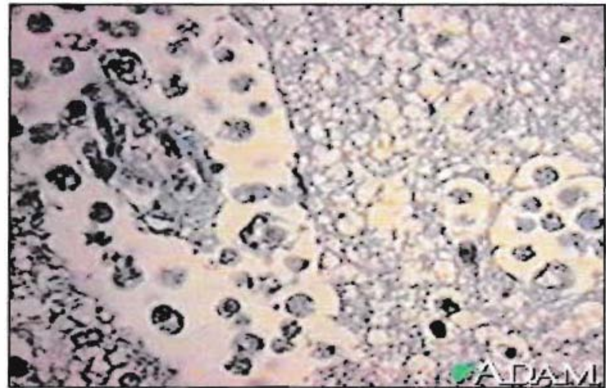


Fig. 17: Amebic brain abscess
http://health.allrefer.com/health/amebiasis-amebic-brain-abscess

Nausea: Nausea is that queasy feeling of unease with which everyone is familiar. It often occurs with or precedes vomiting but both nausea-without-vomiting and vomiting-without-nausea are possible. Nausea is the body's way of reacting to an infection or condition. [41]

Other symptoms of amebiasis includes

- Jaundice
- Sometimes allergic reactions can occur throughout the body, due to release of toxic substances or dead parasites inside the intestines
- Loss of weight and stamina is encountered with person suffering from amebiasis
- Usually symptoms start with diarrhea and pain in right hypochondrium
- Bad smelling stools
- Loss of Appetite
- Weakness or tiredness
- Pain in the right shoulder could occur in chronic condition
- General malaise
- Mental apathy
- Cutaneous lesions in perianal area
- Risk factors for severe amebiasis include:
- Alcoholism
- Cancer
- Malnutrition
- Old age
- Pregnancy
- Recent travel to a tropical region
- Use of corticosteroid medication to suppress the immune system

East West University
LIBRARY
43, Mohakhali, C/A

1.6 Method of Diagnosis

The diagnosis of amebiasis is made in the laboratory by the examination of stool specimens for the presence of trophozoites and cysts. Trophozoites are more likely to be found in liquid stool specimens, while cysts are generally more numerous in formed stools. Important characteristics

used to identify amebae include the number and structure of nuclei, especially the presence and location of chromatin (genetic material found in the nucleus), particularly along the inner nuclear membrane. Most amebic trophozoites have a single nucleus, while the cysts are usually multinucleate. The number of nuclei is characteristic for each species of amebae. All intestinal amebae have karyosomes (clumps of chromatin material found within the nuclei). The size, configuration and location of the karyosomes are distinctive for each ameba and are helpful in identification. [47]

Laboratory Diagnosis

Microscopy

Microscopic techniques employed in a diagnostic clinical laboratory include wet preparation, concentration and permanently stained smears for the identification of *E. histolytica or E. dispar* or *E. moshkovskii* in feces. Microscopic examination of direct saline (wet) mount is a very insensitive method (<10%) which is performed on a fresh specimen. The sample should be examined within 1 hour of collection to search for motile trophozoites which may contain RBCs. However, in patients who do not present with acute dysentery, trophozoites will not contain RBCs. Patients with asymptomatic carriage generally have only cysts in the fecal sample. Microscopy is a less reliable method of identifying *Entamoeba species* than either culture or antigen detection tests. The sensitivity of microscopy can be poor (60%) and confounded with false-positive results due to misidentification of macrophages as trophozoites. [47]

Culture methods

Culture techniques for the isolation of *Entamoeba species* have been available for over 80 years. Culture media include xenic (diphasic and monophasic) and axenic systems. Xenic cultivation is defined as the growth of the parasite in the presence of an undefined flora. The xenic culture of *E. histolytica* was first introduced by Boeck and Drbohlav in 1925 in a diphasic egg slant medium and a modification of this medium is still used today. Different monophasic media that were developed for *E. histolytica* are the egg yolk infusion medium of Balamuth, Jones's medium. Axenic cultivation involves the cultivation of parasites in the absence of any other metabolizing cells. The axenic cultivation of *E. histolytica* was first achieved by Diamond in

1961. Culture of *E. histolytica* can be performed from fecal specimens, rectal biopsy specimens, or liver abscess aspirates.

Intestinal Amebae						
	Entamoeba histolytica	*Entamoeba hartmanni*	*Entamoeba coli*	*Endolimax nana*	*Iodamoeba butschlii*	*Blastocystis hominis*
Trophozoite						No Trophozoite stage
Cyst						

Fig. 18: Characteristic features of trophozoites and cysts of intestinal ameba
http://www.lfhk.cuni.cz/klinmikrob/vyuka/git/parazite.htm

As the liver abscess aspirates of ALA patients are usually sterile (98% cases), addition of a bacterium or a trypanosomatid is necessary before inoculation of ameba into xenic culture. The success rate for culture of *E. histolytica* is between 50 and 70% in reference laboratories. As culture of *E. histolytica* from clinical samples such as feces or liver abscesses has a significant false-negative rate and is technically difficult, it is not undertaken in a routine clinical laboratory. Culture of *E. histolytica* in a clinical diagnostic laboratory is not feasible as a routine procedure as it is less sensitive than microscopy as a detection method. Parasite cultures are difficult, expensive, and labor-intensive to maintain in the diagnostic laboratory. [21]

Isoenzyme analysis

The pioneering work of Sargeaunt et al. demonstrated that isoenzyme analysis of cultured ameba would enable the differentiation of *Entamoeba species*. An isoenzyme (zymodeme) is defined as a group of ameba strains that share the same electrophoretic pattern and mobilities for several enzymes. Zymodemes consist of electrophoretic patterns of malic enzyme, hexokinase, glucose phosphate isomerase, and phosphoglucomutase isoenzyme. Isoenzyme analysis of cultured amoeba enables differentiation of *E. histolytica* from *E. dispar* and was considered the gold standard for diagnosing amoebic infection prior to development of newer DNA-based techniques. [21]

Zymodeme analysis has a number of disadvantages including

- ✓ The difficulty of performing the test
- ✓ It is a time-consuming procedure and relies on establishing the amoeba in culture, with a large number of cells needed for the enzyme analysis
- ✓ This process is not always successful. The cultivation of amoeba may lead to selection bias, and one species or strain may outgrow the other, which is not desirable when studying zymodemes.
- ✓ Furthermore, the amoebic cultures and therefore isoenzyme analyses are negative for many microscopy-positive stool samples
- ✓ Zymodeme analysis is not easily incorporated into routine clinical laboratory work because of the expertise required to culture the parasites, the complexity of the diagnostic process. [21]

Antibody detection tests

Many different assays have been developed for the detection of antibodies including indirect Hem agglutination (IHA), Latex agglutination, Immunoelectrophoresis, Counterimmunoelectrophoresis (CIE), Amoebic gel diffusion test, Immunodiffusion, complement fixation, Indirect immunofluorescence assay (IFA), and Enzyme-linked immunosorbent assay (ELISA). A variety of antibody assays for detection of *E. histolytica* antibodies in human serum are also commercially available. A commercial test kit, CellognostR Reagents for diagnosis of invasive amebiasis by Indirect Hem agglutination (IHA) served as the

standard assay tool. Enzyme-Linked Immunosorbent Assay (ELISA), flat-bottomed wells of nuclonR polystyrene microtiter plates were sensitized with *E. histolytica* Ag overnight at refrigeration temperature. Test and control sera were added to the wells and incubated at 37°C for one hour. Enzyme labeled antihuman immunoglobulin, gamma chain specific, peroxidase conjugate was added to the first two reactants, then the substrate, 2,2 Azino-bis (3 ethylbenzthiazoline-6-sulfonic acid). Plates were incubated for one hour at ambient temperature. A positive titer was indicated by a color change in the substrate as it was oxidized by the enzyme. Since the formation of an Ag-Ab complex (i.e., amoeba-antibody-antihuman gamma globulin-peroxidase conjugate) is specific and irreversible the presence of anti-ameba antibody would be indicated by a positive enzyme substrate reaction, which is proportional to the antibody concentration in the test serum. [42]

Antigen detection tests

Several investigators have developed ELISAs for the detection of antigens in fecal samples. These antigen detection tests have a sensitivity approaching that of stool culture and are rapid to perform. Antigen-based ELISA kits that are specific for *E. histolytica* use monoclonal antibodies against the Gal/GalNAc-specific lectin of *E. histolytica* or monoclonal antibodies against serine-rich antigen of *E. histolytica.* The *E. histolytica* TechLab kit was designed in 1993 to detect specifically *E. histolytica* in feces. This antigen detection test captures and detects the parasite's Gal/GalNAc lectin in stool samples. The lectin is conserved and highly immunogenic, and because of the antigenic differences in the lectin of *E. histolytica* and *E. dispar*, the test enables specific identification of the disease-causing *E. histolytica.* The level of detection of amebic antigens is quite high, requiring approximately 1,000 trophozoites per well. However, this test suffers from the disadvantage that the antigens detected are denatured by fixation of the stool sample, therefore limiting testing to fresh or frozen samples. Nevertheless, this test has demonstrated good sensitivity and specificity for detection of *E. histolytica* antigen in stool specimens of people suffering from amebic colitis and asymptomatic intestinal infection. The TechLab ELISA for detection of *E. histolytica* antigen in stool specimens from people suffering from diarrhea was shown to have an excellent correlation with nested PCR, and in other studies this test was found to be more sensitive (80 to 94%) and specific (94 to 100%) than microscopy and culture. In Bangladesh, 96% and 100% of patients with ALA had detectable levels of lectin

antigen in their serum and liver abscess pus samples, respectively, before treatment with metronidazole. However, the sensitivities of this method were only 33% and 41%for serum and liver abscess pus, respectively, after a few days of treatment with metronidazole, which is probably associated with a decrease in the amount of antigen in the serum or pus following therapy. Results of antigen detection using both the TechLab kits suggest that more specific and sensitive diagnostic tests, such as PCR, are needed to establish the actual worldwide distribution of *E. histolytica* and *E. dispar*. Detection of specific antigens of *E. histolytica* and *E. dispar* in feces by ELISA could be useful for clinical and epidemiological studies where molecular assays cannot be used. Importantly, of the four diagnostic methods, i.e., antigen detection, antibody detection, microscopy, and isoenzyme analysis, only antigen detection using ELISA is both rapid and technically simple to perform and can be used in laboratories that do not have molecular facilities, thus making it appropriate for use in the developing world, where amebiasis is most prevalent. In all cases, the combination of serological tests with detection of the parasite (by antigen detection or PCR) offers the best approach to diagnosis. The advantage of this test is that it can be performed on fresh, frozen or Cary-Blair specimens but not on formalin-fixed fecal samples. [21]

Immunochromatographic assays

The Triage parasite panel (TPP) is the first immunochromatographic assay for the simultaneous detection of antigens specific for *Giardia lamblia*, *E. histolytica*, *E. dispar*, and *Cryptosporidium parvum*. The immunochromatographic strip used in this assay is coated with monoclonal antibodies specific for the 29-kDa surface antigen (*E. histolytica/E. dispar*), alpha-1-giardin (*G. lamblia*), and protein disulfide isomerase (*C. parvum*). By using specific antibodies, antigens specific for these organisms from the stool samples are captured and immobilized on a membrane. A high sensitivity (96% to 100%) and specificity (99.1% to 100%) of the TPP kit compared to microscopy (stool ovum and parasite examination) for *E. histolytica/E. dispar* were reported. The advantage of the TPP method is that it can be performed in approximately 15 min with fresh or frozen, unfixed human fecal specimens. The TPP provides diagnostic laboratories with a simple, convenient alternative method for performing simultaneous, discrete detection of *Giardia-*, *Cryptosporidium* and *E. histolytica/E. dispar*-specific antigens in patient fecal specimens. [21]

East West University LIBRARY 43, Mohakhali, C/A

1.7 Treatment of Amebiasis

The Dauphin de France was the first known patient of amebiasis who was treated by an extract of the root of the ipecacuanha plant. Emetine is the active alkaloid of ipecacuanha was introduced for the treatment of amebiasis in 1912 and was the standard drug used in this disease for about 50 years. Unfortunately, toxicity hampers its usefulness that justified the search for other drugs. Because of the various aspects of amebiasis, one has to face several therapeutic problems in drug treatment:

- Reach the amebae in the colonic lumen
- Destroy the parasite in the tissues, both locally in the wall of the colon and in other organs where the parasite may have migrated
- Prevent the formation of cysts, in order to avoid the spread of the disease
- Reduce as much as possible the duration of treatment, bearing in mind that the disease occurs mainly in rural areas, where people are not inclined to take drugs for more than a few days
- Administer drugs with as few side effects as possible
- Administer inexpensive treatment

If all these conditions can be fulfilled then that would be ideal drug to treat the various forms of amebiasis. Halogenated hydroxyquinolines such as entero-vioforme were improvements over emetine in that they have a better luminal amebicide action, better tolerated and are easily administered. On the other hand, they have no action against tissue amebae and therefore their use is limited. Moreover, the excessive use of drugs of this type can lead to serious toxic effects, as seen in Japan with the SMON disease. Arsenicals, such as milibis, have been extensively used in intestinal amebiasis. They are well-tolerated and good amebicides. But they do not reach the parasites in the tissues and therefore can only be used as an adjuvant treatment. Apparently these compounds can prevent the formation of cysts by destroying the trophozoites. Arsenicals require a treatment of 10 days and the drugs are eliminated very slowly from the body. [35]

Medical Treatments

- Diloxanide furoate is a good luminal amebicide which devoid of toxic effects and able to eliminate trophozoites and cysts from the colon.

- Among the antibiotics, Paromomycin is the only one which is directly amebicidal both in vitro and in vivo. It may be useful in severe amebic dysentery with marked bacterial over infection.
- Emetine derivative, 2-dehydroemetine achieved a major breakthrough in the treatment of the disease. The main advantage of this compound over emetine is its lower toxicity with much less accumulation in the tissues. A seven-day treatment with 2-dehydroemetine of 1 mg/kg body-weight destroys the parasite both in the colonic lumen and in the tissues.
- In extra-intestinal amebiasis chloroquine usually in association with emetine has a good amebicidal action and has been used with success not in amebic liver abscess.
- The most significant advance in the treatment of amebiasis is the discovery of amebicidal properties of nitroimidazole derivatives. In 1966 Powell et al reported the success of metronidazole in both amebic dysentery and liver abscess. The drug is extremely active against *E. histolytica.* In culture, the morphology of the organism is altered markedly within 6 to 20 hours by concentrations of 1 to 2 μg/ml of metronidazole. Within 24 hours all organisms are for killed.
- Metronidazole given orally for 5 to 10 days at 2.25 g daily in amebic dysentery and 1.5 g to 2.25 g daily for 5-10 days in amebic liver abscess. More recently, Bunnag et al in Bangkok successfully treated amebic liver abscess with 2.4 g of metronidazole given orally for a single day.
- Tinidazole and Ornidazole are most promising. Tinidazole given for three days at dosage of 2 g a day for adults and about 60 mg/kg bodyweight a day children achieves a parasitic cure rate of 83% to 96%. The compound is well absorbed and has a half-life of 12 hours.
- A newly developed nitroimidazole derivate, ornidazole (Tiberal) also shows excellent anti-protozoal action at a low dose against *Trichomonas vaginalis*, *Giardia lamblia* and *Entamoeba histolytica.* Ornidazole is well tolerated but not devoid of side effects. From comparative studies between metronidazole, tinidazole and ornidazole, the latter two compounds seem to be superior in terms of efficacy in the management of intestinal amebiasis. Because these drugs have shorter duration of action and Side-effects are comparable. Ornidazole has a longer half-life which is an advantage and its efficacy in a one-day treatment in amebic liver abscess is documented, which is not the case with tinidazole. [35]

Based on recent publications the usual drug regimens recommended are:

Table 1: Different drugs dosage regimens

Presentation	Drng	Adult Dosage	Efficacy
Asymptomatic cyst passage (luminal infection only)	Diloxanide furoate	500 mg t.i.d., 10 days	87-96%
	Iodoquinol (Diiodohydroxyquin)	650 mg t.i.d., 20 days	95%
	Paromomycin	500 mg t.i.d, 10 days 30 mg/kg/day in 3 doses, 5-10 days	85-90%
Invasive Rectocolitis	Metronidazole	750 mg t.i.d., 5-10 days 50 mg/kg, 1 dose	90+% 86%
	Tinidazole	50 mg/kg q.d., 3 days	
Amoebic Liver Abscess	Metronidazole	750 mg t.i.d., 5-10 days 2.4 g q.d., 1-2 days	95%
	Tinidazole	2 g p.o.	
	Ornidazole	2 g p.o.	

Source: http://entamoeba.lshtm.ac.uk/treat.htm

Surgical Care

Surgical intervention is required for the treatment of acute abdomen due to perforated amebic colitis, massive GI bleeding, or toxic megacolon. Toxic megacolon is rare and is typically associated with the use of corticosteroids. Surgical attempts to correct amebic bowel perforation or peritonitis should be avoided, although some patients may benefit from peritoneal lavage.

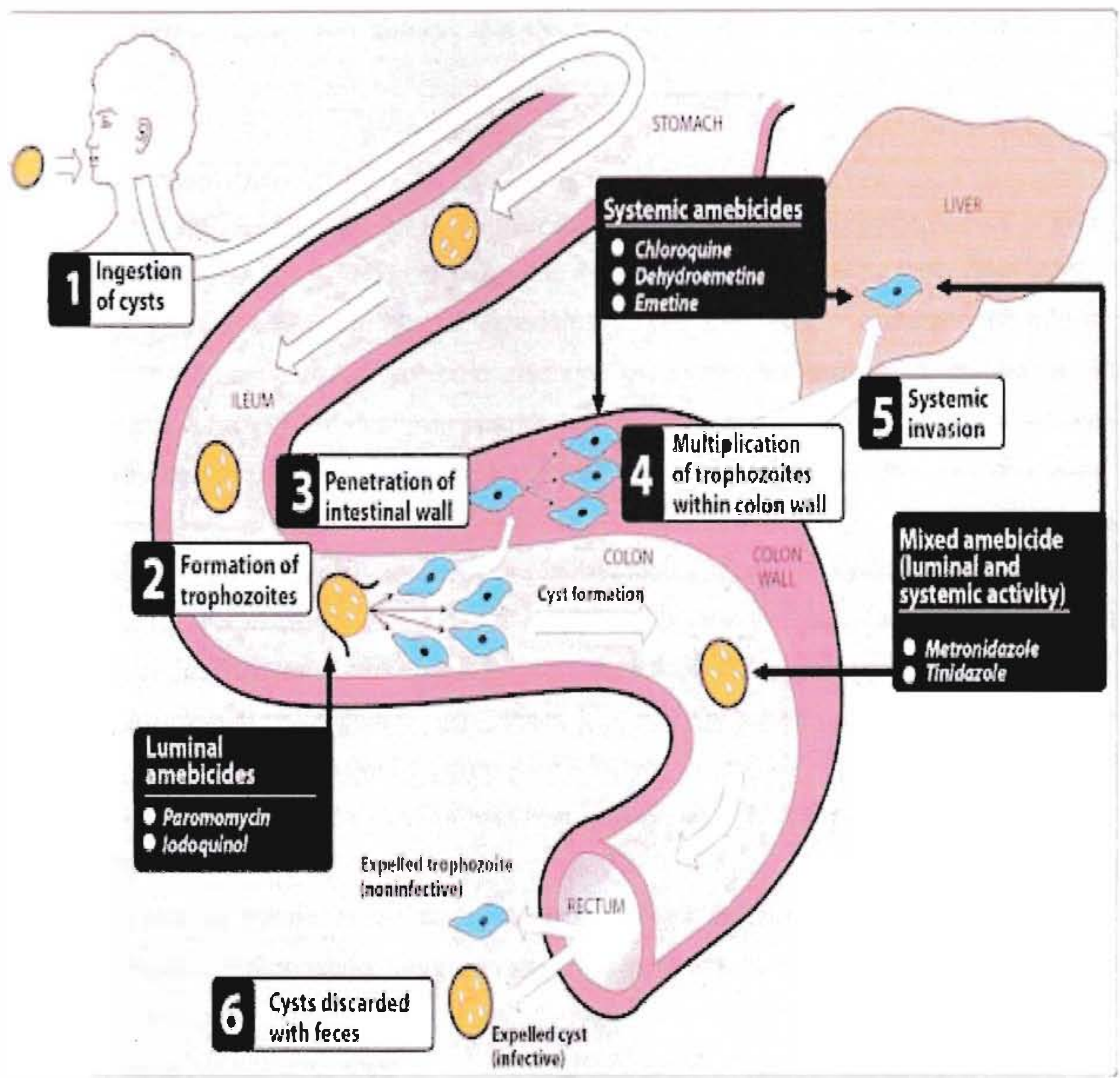


Fig.19: Different stages of *E. histolytica* inside the human body, showing the site of action of amebicidal drugs *(Harvey and Champe)*

Unlike pyogenic liver abscess, amebic liver abscess generally responds to medical therapy alone and drainage is seldom necessary. When necessary, imaging-guided percutaneous treatment (needle aspiration or catheter drainage) has replaced surgical intervention as the procedure of choice for reducing the size of an abscess. The indications for drainage of amebic liver abscess include the following:

- Presence of left-lobe abscess (>10 cm in diameter)

- Impending rupture and abscess that does not respond to medical therapy within 3-5 days.[15]

Ayurvedic Treatments

Kutaja is the medicine of choice for treating amebiasis. One teaspoon of powdered bark is given three times a day. Patients suffering from amebic dysentery are also liable to suffer from chronic constipation and so should take two tablespoonfuls of flea seed husk at bedtime with milk or buttermilk. In chronic cases, Ayurvedic medicine prescribes Rasaparpati, a preparation of mercury and sulphur (150 mg daily in chronic cases). During treatment the taking of salt and water should be strictly prohibited and diet should be limited to milk, boiled rice and sugar. Other remedies:

- ✓ *Takrarishta*: Seeds of Ajwain, Chebulic myrobalan, Emeblic myrobalan, black pepper powder, special types of salts like Saindhav and Sourvachal salts should be mixed with buttermilk. This buttermilk should be used when fermentation is complete.
- ✓ *Dadimavaleha*: Pomegranate fruit, black pepper, long pepper, ginger, sugar should be mixed together and cooked. 5 grams, twice daily.
- ✓ *Bilvavaleha*: Prepared a paste of bael fruit in same way as Dadimavaleha. 5 grams, twice daily.
- ✓ Combine the powder of bael root, ginger powder and long pepper. 500 mg twice daily.
- ✓ A decoction of coriander seeds and ginger powder is a good anti dysenteric remedy. 15 ml three times daily.
- ✓ *Kutajaghan vati* (the tablets of Kurchi seeds). Two tablets three times a day. [15]

Homeopathic Treatments

Homeopathy can immediately control all the symptoms of amebiasis like diarrhea, urgency, mucus in the stools and abdominal pain. Homoeopathy can also see that the *Entamoeba histolytica* remains only as a commensal in the large intestine without progressing to cause its symptoms and prevent further complications like amoebic liver abscesses etc. Homoeopathy for amebiasis is as wholesome as one would want. Homeopathic medicines commonly used in cases of amebiasis are *Aloes, Ars alb, Baptisia, Cinchona, Colocynth, Ipecac, Kali Birch, Lycopodium, Mer cor, Nux vomica, Podophyllum, Pulsatilla, Rhus tox, Sulphur, Trombidium, Thuja.*[15]

Home Remedies for Amebiasis

- ✓ Neem: Equal quantities of dried neem leaves and turmeric powder are taken and mix with mustard oil. This mixture is applied on the body and leaves it for an hour. Later, have bath with lukewarm water. This is the most beneficial remedy because neem acts as an antibiotic.
- ✓ Apricot: Juice from apricot leaves is extracted and applied on the infected areas. This is the most effective remedy for treating amebiasis.
- ✓ Drumstick: 400 – 500 grams of drumstick leaves are taken and juice is extracted. Equal amounts of sesame seed oil are mixed and it is heated until water evaporates. The mixture is cooled and stored. This mixture is applied on the infected areas daily. Both drumstick leaves and sesame seed oil have anti-microbial properties.
- ✓ Peepul: Bark of peepul tree is taken and dried. The bark is grinded to make fine powder and strain through fine sieve. This powder is used on the lesions of amebiasis.
- ✓ Bael fruit: An unripe bael fruit is roasted on open fire and pulp is removed. This is taken along with jaggery or sugar. It is an excellent remedy for treating amebiasis. [15]

Nutritional Therapy

- Sugars and simple carbohydrates like those found in refined foods are avoided
- Dairy products are avoided
- Caffeine and alcohol should eliminate
- Raw garlic, pumpkin seeds, pomegranates, beets, and carrots, all of which have anti-parasitic properties should be taken
- Should drink a lot of water to promote good bowel elimination. [15]

1.8 Drug Resistance in Amebiasis

Amebiasis caused by *Entamoeba histolytica* is a major public health problem in developing countries. The indiscriminate use of antiamebic drugs can result in increased minimum inhibitory concentration (MIC) values against *Entamoeba* species and treatment failure may emerge as an important public health problem. Poverty, ignorance, overcrowding, poor sanitation and malnutrition favour transmission and increased disease burden. Prevalence varies from country to country. The recommendation is that *E. histolytica* should be specifically identified, treated

with antiamebic drugs. In individuals with only *E. dispar*, the treatment is unnecessary. Treating asymptomatic individuals indiscriminately may lead to drug resistance. [5]

***In Vitro* Drug Sensitivity**

To understand the magnitude of drug resistance, drug sensitivity of clinical isolates of *E. histolytica* is important. Minimum inhibitory concentration (MIC) of metronidazole ranging from 12.5-25 μm. Adagu et al12 have shown the mean 50 per cent inhibitory concentration (IC50) value to metronidazole as 18.47 μm for the most susceptible isolates of *E. histolytica* with a >30 μm value as the cut-off value for resistance. [5]

Drug Resistance Mechanisms in *E. histolytica*

Parasite may evade drug action by hiding in sanctuaries. So far the mechanisms of drug resistance hypothesized in protozoan parasite are decrease of drug uptake because of loss of a transporter required for uptake, the efflux of drugs from the parasite either by the P-glycoproteins (Pgp) or by ATPases, the alteration of drug target, and loss of drug activation. Metronidazole and related nitroimidazole, tinidazole, are the only effective drugs for the treatment of *trichomoniasis* and *giardiasis*. Tumour cells become resistant to simple chemotherapeutic drugs in vitro. The drug resistance is usually due to increased efflux of the drug from the tumour cells, which is energy dependent and is inhibited by calcium ion channel blockers. [5]

1.9 Prevention of Amebiasis

Amebiasis is a social problem and its definitive eradication depends on the improvement of public health measures, adequate sanitation and, better health education. The development of a vaccine would be the most economical strategy for the prevention of amebic infection. The great challenge to vaccine development includes an incomplete understanding of immunity in humans and in animal models. Although a vaccine is not yet commercially available, there has been continuous progress in this field. [44] Amebiasis can be prevented by practicing good hygiene and by using caution before eating food or drinking water from an unknown source. Some general guidelines are:

- ✓ To reduce transmission of amebiasis, wash hands thoroughly with soap and hot running water for at least ten seconds:
    - Before preparing food
    - Before eating
    - After using the toilet or changing diapers
    - After smoking
    - After using a tissue or handkerchief
    - After changing clothes or bedding soiled with stool
    - After touching or petting animals
- ✓ Do not drink untreated water from a surface water supply such as a pond, lake or stream. The water may appear clear but it could be contaminated with human waste. If untreated water is all that is available, it can be boiled for 1 minute to make it safe. Many water purification tablets are not effective against *E. histolytica.*
- ✓ Unfortunately, Ordinary chlorination of water cannot be relied upon kill amebic cyst, but chlorination plus proper filtration seem to be effective. A special iodine tablet known as Globaline is useful for small scale use for household drinking water. When in doubt, boil all drinking water, for this will not only kill the amebic cyst, but another organism present.
- ✓ Thoroughly wash all fruits and vegetables that are eaten raw. Disinfectant dips for fruits and vegetable are of unproven value in preventing transmission of E.histolytica. Thorough washing with potable water and keeping fruits and vegetables dry may help; cysts are killed by desiccation, by temperatures above 50°C (122°F) and by irradiation.
- ✓ Travelers to areas of poor sanitation should be aware of the risk of this infection and should only drink water or use ice from a source known to be free of contamination. They should avoid eating unpasteurized milk or dairy products, which can be contaminated with unclean water. They should not eat uncooked fruits unless they can peel them themselves and not eat salads or raw vegetables.
- ✓ Men who have sex with men should be aware of the risk of infection and may wish to avoid sexual practices that involve fecal-oral contact. If symptoms occur, advice from a health care provider should be sought. Infected persons should advise contacts to be evaluated and treated if necessary to minimize the risk of spread and re-infection.

- ✓ The internal temperature of reheated food reaches at least 75°C.
- ✓ Should avoid sharing towels or face washers.
- ✓ Should drink only packaged drinks, boiled water or chlorinated and filtered water.
- ✓ Drinks containing ice are avoided.

1.10 Amebiasis in Bangladesh

NIAID currently funds major research projects in Bangladesh, which are primarily focused on endemic diseases, such as cholera, amebiasis, and cryptosporidiosis; and on enteric pathogens, such as *Vibrio cholera* and *Entamoeba histolytica*. Outbreaks and transmission of viruses, such as henipaviruses and enteroviruses, are also subjects of NIAID-funded research. One of the leading collaborating institutions is the International Centre for Diarrheal Disease Research in Dhaka, Bangladesh (ICDDRB).The contribution of amebiasis to the burden of diarrheal disease in children and the degree to which immunity is acquired from natural infection were assessed in a 4-year prospective observational study of 289 preschool children in an urban slum in Dhaka, Bangladesh. *Entamoeba histolytica* infection was detected at least once in 80%, and repeat infection in 53%, of the children who completed 4 years of observation. Annually there were 0.09 episodes/child of *E. histolytica*-associated diarrhea and 0.03 episodes/child of *E. histolytica*-associated dysentery. Amebiasis was a substantial burden on the overall health of the cohort children. Protection from amebiasis was associated with a stool anti-CRD IgA response. The challenge of producing an effective vaccine will be to improve upon naturally acquired immunity, which does not provide absolute protection from reinfection. Both bacillary dysentery and amoebic dysentery are highly endemic in Bangladesh and is the cause of extensive morbidity and mortality, particularly in children with attacks of bacillary dysentery. Approximate yearly cases of bacillary dysentery in Bangladesh in a typical year is estimated to be between 2,00,000 and 3,00,000 while the number for amoebic dysentery is estimated to be between 25,000 and 50,000. Incidence of amebiasis is high where sewerage system is poor and in villages of developing countries where open air defecation is common. The poor socio-economic condition of Bangladesh and the practice of unsafe faeces disposal and, in villages, the practice of open-air defecation, have made amebiasis an endemic disease of high prevalence throughout the year. In many developing countries including Bangladesh, malnutrition involving both carbohydrate and protein deficiency is common. [23]

1.11 Drugs Commonly Used in Amebiasis

Ornidazole

Ornidazole belongs to the 5-nitroimidazole group of drugs and is a potent anti protozoal and anti microbial agent for the treatment of infections associated with T. vaginalis, *E. histolytica, G. lamblia* and certain anaerobic bacteria.

Ornidazole

Physicochemical Properties

Ornidazole has a heterocyclic structure consisting of a nitroimidazole-based nucleus with a 2-hydroxy-3- chloro-propyl group in position 1 and a methyl group in position 2. It is synthesized from 5-nitroimidazole derivatives. Ornidazole is known chemically as 1-(2-hydroxy-3-chloropropyl)-2-methyl-5-nitroimidazole ($C_7H_{10}ClN_3O$). Its molecular weight is 219.63 (C 38.28%, H 4.59%, Cl 16.14%, and N 19.13%, O 21.85%). Ornidazole is a white to yellowish microcrystalline powder. It's melting point between 358K to 360 K. Its 1% aqueous solution has a pH of approximately 6.6. Ornidazole is soluble in water, ether, ethanol and chloroform (2.6, 2.4, over 50, over 50%, respectively). [19]

Mechanism of Action

The anti microbial activity of ornidazole is due to the reduction of the nitro group to a more reactive amine that attacks microbial DNA, brings about loss of helical structure of DNA and subsequent DNA breakage, thus inhibiting further synthesis and causing degradation of existing DNA of the microbes. Nitroimidazole are thought to produce their bactericidal activity through four phases:

(I) Entry into the bacterial cell

(II) Nitro group reduction

(III) Action of the cytotoxic by products

(IV) Production of inactive end products [19]

East West University
EAST WEST
LIBRARY
43, Mohakhali, C/A

Pharmacokinetics

Ornidazole is readily absorbed from the gastro-intestinal tract and peak plasma concentrations of about 30 mcg per ml have been achieved within 2 hours of a single dose of 1.5 g, falling to about 9 mcg per ml after 24 hours and 2.5 mcg per ml after 48 hours.

The plasma elimination half-life of ornidazole is 12 to 14 hours. Less than 15% is bound to plasma proteins. It is widely distributed in body tissues and fluids, including the cerebrospinal fluid.

Ornidazole is metabolized in the liver and is excreted in the urine, mainly as conjugates and metabolites, and to a lesser extent in the faeces; 85% of a single oral dose has been reported to be eliminated within 5 days, 63% in the urine and 22% in the faeces.[3] Biliary excretion may be important in the elimination of ornidazole and its metabolites. [29]

Indications and Usage

- Used in the treatment of Bacterial vaginosis (non-specific vaginitis).
- Trichomoniasis. Genitourinary infections in women and men due to Trichomonas vaginalis.
- Amebiasis. All intestinal infections due to Entamoeba histolytica, including amoebic dysentery.
- All extra intestinal forms of amebiasis, especially amoebic liver abscess.
- Giardiasis (lambliasis).
- Infections due to anaerobic bacteria. Treatment of infections such as septicaemia, meningitis,
- peritonitis, postoperative wound infections, puerperal sepsis, septic abortion, and endometritis,
- With demonstrated or suspected involvement of susceptible bacteria (see Properties and Effects).

- Prophylaxis during surgical interventions, particularly those involving the colon, and in
- Gynecological operations. [40]

Contraindications

It shows hypersensitivity to imidazoles.

Warnings and Precautions

Special precautions are required in case of ataxia, vertigo, mental confusion and patients with neurological diseases. There is need for adjustment of dosage and dosage interval in patients with hepatic impairment. Modification of the usual dosage is not necessary in patients of renal failure however; additional post hemodialysis dose may be required in patients undergoing this procedure.

Adverse Reactions

The most frequently encountered side effect is dizziness, alone or in combination with other adverse reactions. The other side effects occurring to a lesser extent are nausea, pyrosis, intestinal spasms and metallic taste. Vertigo, fatigue and other discomforts such as loose stools, insomnia, skin rash and headache have also been reported. Taste disturbances, abnormal liver function tests and skin reactions have been observed.

Drug interactions

In contrast to other nitroimidazole derivatives, ornidazole does not inhibit aldehyde dehydrogenase and is therefore not incompatible with alcohol. However, ornidazole potentiates the effect of coumarin type oral anticoagulants. The dosage of the anticoagulant has to be adjusted accordingly. Ornidazole prolongs the muscle-relaxant effect of vecuronium bromide.

Over dosage and Treatments

Over dosage may cause exacerbation of all the pharmacological side effects of ornidazole.

Dosage and Administration

- Ornidazole is given by mouth in tablets after food, or intravenously. When given intravenously, solutions of ornidazole should be diluted to 5 mg or less per mL and 100 or 200 mL infused over 15 to 30 minutes. It has also been given by vaginal pessary.
- In amebiasis, 500 mg of ornidazole is given twice daily by mouth for 5 to 10 days. Patients with amebic dysentery may be given 1.5 g as a single daily dose for 3 days. In severe amebic dysentery and amebic liver abscess, ornidazole may be given by intravenous infusion in a dose of 0.5 to 1 g initially, followed by 500 mg every 12 hours for 3 to 6 days.
- In giardiasis, 1 or 1.5 g of drug is given by mouth as a single daily dose for 1 or 2 days12, 42-44.
- In trichomoniasis, a single dose of 1.5 g is given by mouth or 1 g by mouth together with 500 mg vaginally is given; alternatively, a 5-day course of ornidazole 500 mg twice daily by mouth, with or without 500 mg vaginally, is also used. Sexual partners should be treated concomitantly.
- For the treatment of anaerobic bacterial infections, ornidazole is given by intravenous infusion in an initial dose of 0.5 to 1 g, followed by 500 mg every 12 hours for 5 to 10 days; oral therapy with 500 mg every 12 hours should be substituted as soon as possible.
- For the prevention of post-operative anaerobic bacterial infections, 1 g is given by intravenous infusion about 30 minutes before surgery.
- Crohn's disease is a chronic disorder that causes inflammation of the digestive or gastrointestinal tract. Ornidazole is an effective and safe drug for the treatment of active Crohn's disease. Ornidazole is given to patients in doses of 20 mg/kg BW (body weight) per day in two separate doses for the treatment.
- Ornidazole is one of the most frequently used antibiotics for curing Helicobacter pylori infection. In the treatment, 500 mg ornidazole is used with 30 mg lansoprazole and 1 g amoxicillin. [29]

Metronidazole

It is an oral synthetic antiprotozoal, amebicide and antibacterial agent. Chemically it is known as 1-β-hydroxyethyl)-2-methyl-5-nitroimidazole. It is in the group of nitroimidazole antibiotic.

Metronidazole and related nitroimidazoles are active in vitro against a wide variety of anaerobic protozoal parasites and anaerobic bacteria. [27]

Metronidazole.

This drug is effective against both intestinal and hepatic amebiasis. It has also been found of use in the treatment of such other protozoal diseases as giardiasis and balantidiasis. The 2-hydroxy metabolite of metronidazole is active; other metabolites are inactive. [6]

Mechanism of Action

Metronidazole is extremely useful in the treatment of extraluminal amebiasis and it kills the trophozoites but does not kill the cysts of *E. histolytica.* [33]

It is a prodrug that is activated by reduction of the nitro group by susceptible organisms. Unlike their aerobic counterparts, anaerobic and microaerophilic pathogens such as *T. vaginalis, E. histolytica,* and *G. lamblia* and anaerobic bacteria contain electron transport components that have a sufficiently negative redox potential to donate electrons to metronidazole. Electron transfer forms a highly reactive nitro radical anion that kills susceptible organisms by radical-mediated mechanisms that target DNA. Metronidazole is catalytically recycled; loss of the active metabolite's electron regenerates the parent compound. Increasing levels of O2 inhibit metronidazole-induced cytotoxicity because O2 competes with metronidazole for electrons generated by energy metabolism. Thus, O2 can both decrease reductive activation of metronidazole and increase recycling of activated drug. In susceptible organisms, pyruvate decarboxylation, catalyzed by pyruvate: ferredoxin oxidoreductase (PFOR) produces electrons that reduce ferredoxin, which then catalytically donates electrons to biological electron acceptors or to metronidazole. [27]

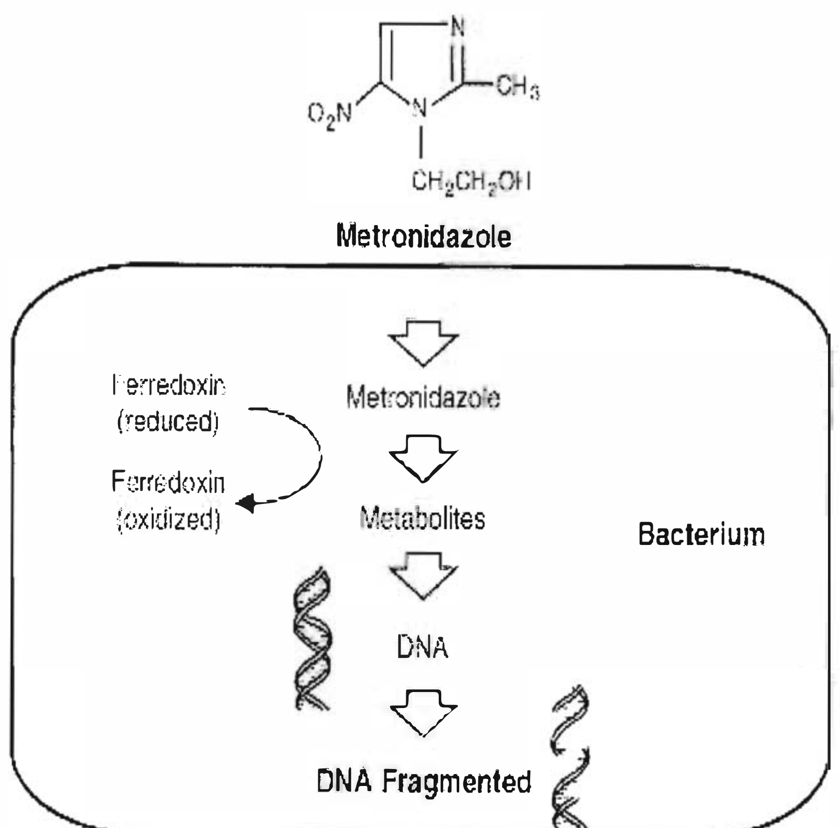


Fig. 20: Mechanism of action of metronidazole
http://microblog.me.uk/nodepage

Nitazoxanide

It is a synthetic nitrothiazolyl-salicylamide derivative and an antiprotozoal agent for oral administration. Nitazoxanide is a light yellow crystalline powder. It is poorly soluble in ethanol and practically insoluble in water. Chemically, nitazoxanide is 2-acetyloxy-*N*-(5-nitro-2-thiazolyl) benzamide. The molecular formula is $C_{12}H_9N_3O_5S$ and the molecular weight is 307.3.

Nitazoxanide

Nitazoxanide is designated by the U. S. Food and Drug Administration (FDA) as an orphan drug. It has been used to treat *G. intestinalis* that is resistant to metronidazole and albendazole. It is indicated for treatment of diarrhea caused by Cryptosporidium parvum and Giardia lamblia in children. [45]

Mechanism of Action

Nitazoxanide appears to interfere with the PFOR enzyme-dependent electron-transfer reaction. This reaction is essential in anaerobic metabolism. Nitazoxanide does not appear to produce DNA mutations. [27]

Diloxanide Furoate

It is an anti protozoal drug and is used in the treatment of infection associated with E. histolytica and other protozoal infections. It refers to the group of luminal amebicides.

Diloxanide Furoate

Diloxanide furoate is a dichloroacetamide derivative. Its molecular weight is 328.2 and the chemical formula is $C_{14}H_{11}Cl_2NO_4$. It is chemically known as 4-[(dichloroacetyl)(methyl)amino]phenyl furan-2-carboxylate. Non polar esters of Diloxanide are more potent than polar ones (Block and Beale, 2004). This drug can be used in asymptomatic, mild to moderate, severe intestinal infection and also in hepatic abscess. [33]

Secnidazole

Secnidazole is a synthetic derivative of the nitroimidazole series of anti protozoal drugs. It belongs to the group of tissue amebicide and chemically known as 1-(2-methyl-5-nitro-1*H*-imidazol-1-yl) propan-2-ol. Its molecular formula and molecular weight is $C_7H_{11}N_3O_3$ and 185.2 respectively.

Secnidazole

Tinidazole

Tinidazole is an anti parasitic drug and is used widely in protozoal infection. It may be a therapeutic alternative in the setting of metronidazole tolerance. It is a second-generation 2-methyl-5-nitroimidazole.

Tinidazole is chemically known as 1-(2-ethylsulfonylethyl)-2-methyl-5-nitro-imidazole and the molecular formula is $C_8H_{13}N_3O_4S$. Its molecular weight is 247.3.

Tinidazole

Paromomycin

Paromomycin is the drug of aminoglycoside antibiotic group. It is used orally to treat *E. histolytica, cryptosporidiosis, and giardiasis.* It is the drug of choice for intestinal colonization with *E. histolytica* and is used in combination with metronidazole to treat amebic colitis and amebic liver abscess. [27] It inhibits protein synthesis by binding to 16S subunit of ribosomal RNA.[33] Paromomycin sulfate is designated chemically as *O*-2,6-Diamino-2,6-dideoxy-β-L idopyranosyl-(1→3)-*O*-β-D-ribofuranosyl-(1→5)-*O*-[2-amino-2-deoxy-α-D-glucopyranosyl

(1→4)]-2-deoxystrepta-mine sulfate (salt). The molecular formula is $C_{23}H_{45}N_5O_{14} \cdot xH_2SO_4$, with a molecular weight of 615.64 (base).

Paromomycin

Iodoquinol

It is a halogenated hydroxyquinoline that is effective against organisms in the bowel lumen but not against trophozoites in the intestinal wall or extra intestinal tissue. It molecular formula is $C_9H_5I_2NO$ and molecular weight is 396.9. Iodoquinol is an alternative drug for the treatment of asymptomatic or mild to moderate intestinal amebiasis. Although it is not effective against ameba's or extra intestinal forms of the disease, including hepatic amebiasis, it is used to treat concurrent intestinal infection. [33]

Iodoquinol

roquine

roquine is 4-aminoquinolone derivative and is widely used against the malaria as well as cidal drug. Chemically it is known as 7-chloro-4-[[4- (diethylamino)-1-methylbutyl]] quinoline phosphate and the molecular formula is $C_{18}H_{26}ClN_3$. Its molecular weight is

Chloroquine

ine is directly toxic to *E. histolytica* trophozoites and is highly concentrated within the ing it effective for amebic liver abscess. It is used only when metronidazole or another zole is either contraindicated or unavailable. Chloroquine is not effective against amebiasis because it only attains low concentrations in the colon; patients receiving e for amebic liver abscess also should receive paromomycin or iodoquinol to testinal colonization. [27]

another agent for the treatment of amebiasis. It inhibits protein synthesis by blocking ation. It is concentrated in the liver, where it persists for a month after a single dose. metabolized and excreted, and it can accumulate. Dehydroemetine is less toxic than

Emetine

Emetine is produced from the ipecac root. It is chemically known as (2*S*,3*R*,11b*S*)-2-{[(1*R*)-6,7-Dimethoxy-1,2,3,4 tetrahydroisoquinolin-1-yl]methyl}-3-ethyl-9,10- dimethoxy-2,3,4,6,7,11b-hexahydro-1*H*-pyrido [2,1-*a*] isoquinoline. Its molecular formula and molecular weight is $C_{29}H_{40}N_2O_4$ and 480.6 respectively.

Objective of the Study

A large number of populations in Bangladesh are suffered from amebiasis caused by *Entamoeba histolytica.* Only limited numbers of drugs are available for the treatment of amebiasis. The indiscriminate use of antiamebic drugs can result in increased minimum inhibitory concentration (MIC) values against *Entamoeba* species, and treatment failure may emerge as an important public health problem. To overcome this problem we have to find out more effective antiamebic drugs. Two brands of Ornidazole, Ornil and Robic may be good choices. Therefore the present study was conducted to evaluate the "*In vitro* sensitivity of Ornil and Robic against clinical isolates of *Entamoeba Histolytica*".

Significance of the Study

Ornil and Robic are two different brands of Ornidazole group, which is a nitroimidazole derivative with activity against protozoa and anaerobic bacteria. It has been used successfully in patients with vaginal trichomoniasis and amebiasis because of its excellent penetration into lipidic tissues, prophylactically for abdominal and gynecological surgery. The mean half-life of elimination from human plasma is 11 to 14 hours. The long serum half-life of ornidazole would permit a more convenient dosage interval. Ornil and Robic have a large spectrum of activity and can be given in different dosage forms. Moreover, these compounds are an effective drug which is relatively safe and produces only minimal side effects

East West University
LIBRARY
43, Mohakhali, C/A

Methods

&

Materials

2. Materials and Methods

2.1 Research Design

The research design is the "*In vitro* Sensitivity study of Robic and Ornil against clinical isolation of *Entamoeba histolytica*".

2.2 Collection of Clinical Isolation

Clinical isolates from patient attending the Out Patient Department of ICDDRB, hospital, attached to the parasitological Laboratory, ICDDRB, and Dhaka, Bangladesh. Clinical isolation of *E.histolytica* were harvested from 24 h old cultures and suspended in a LYI-S-2 medium. Identification no of the clinical isolation of *E.histolytica* was 2759071. Axenic medium consists of liver digest, yeast extract, iron and serum. The parasite count was adjusted to 1.575×10^{5} mL^{-1}.Isolation is usually achieved by growing the species in an environment that was previously sterilized and was thereby rid of contaminating organisms.

The instruments are:

- ✓ Analytical Balance
- ✓ Morter and pestle
- ✓ Vortex machine
- ✓ Micropipettes
- ✓ Eppendrof (1ml and 2ml)
- ✓ Microtiter plate
- ✓ Microscope
- ✓ Haemocytometer
- ✓ Microtips.

2.3 Cultivation of *Entamoeba histolytica*

General Considerations

Three types of culture system will be discussed in this manual for the cultivation of *Entamoeba* species. Xenic cultivation where the parasite is grown in the presence of an undefined flora,

Monogenic cultivation where the parasite is grown in the presence of a single species of associate and Axenic cultivation where the parasite is grown in the absence of any other metabolizing cells. The term polygenic is sometimes erroneously used as a synonym for xenic, however polygenic should refer only to cultures where the identities of all the species present are known. These parasitic protista are all isolated from sources rich in bacteria and fungi. Controlling or eliminating the latter's growth is crucial to success in cultivating the parasites of interest. This is true even in xenic culture, where a balance between the needs of the bacterial flora and the eukaryote is important. [16]

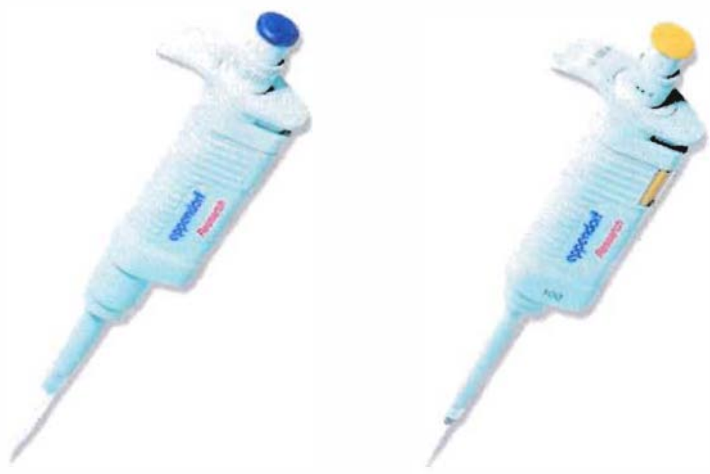


Fig. 21: Micropipette

Fig. 22: Microcentrifuge tube

Unlike *Trichomonasvaginalis* and *Giardia intestinalis* which can readily be established directly into axenic cultures, *Entamoeba histolytica* has never been grown axenically without first being established in culture with other organisms and usually with a complex undefined bacterial flora. Axenization of *E. histolytica* is a laborious and lengthy process where xenic cultures of the organism are first adapted to monoxenic growth, usually with a kinetoplastid flagellate as an associate, before weaning them from a phagocytotic lifestyle to one where the nutrients are obtained largely by pinocytosis. [17]

Historical background

Entamoeba histolytica was first established in culture by Boeck and Drbohlav in 1925 in a diphasic egg slant medium they had developed for isolation of intestinal flagellates. [8]

A modification of this medium (LE) is still in use today. Their success refuted the Promethean view of *E. histolytica* as an obligate tissue parasite. Dobell introduced the use of rice starch as a carbohydrate source, and it remains a component of all media for xenic cultivation to this day. Soluble sugars, were they to be used, would be metabolized too rapidly by the bacteria and would not be available to the amoebae. Several diphasic media were developed subsequently with serum, agar or egg extracts in the slants. [18] Currently the most widely used media for xenic cultivation of *E. histolytica* are the diphasic LE and Robinson's media and the monophasic TYSGM-9. Monoxenic cultivation of *E. histolytica* was first accomplished by Cleveland and Sanders (Cleveland et.al,1930) in a diphasic medium with a single species of bacterium, and the monobacterial Modified Shaffer-Frye medium (MS-F) was widely used at one time. Monoxenic cultivation is of limited use today except as a transitional stage between xenic and axenic cultures. *Crithidiafasciculata* or *Trypanosomacruzi* are the associates of choice for monoxenic cultivation. [17]

2.4 Media for Axenic Cultivation

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

used. TP-S-1 was superseded by TYI-S-33. [17] And this is currently the most widely used medium for axenic cultivation of *E. histolytica.*

Fig. 23: Autoclave **Fig. 24: Laminar air flow machine**

2.5 Media for Xenic Cultivation

Xenic culture will be for identification of the species of *Entamoeba* present in the sample, for example by isoenzyme analysis. Several intestinal species of *Entamoeba* can be found in human who are sufficiently similar to cause diagnostic confusion and all can grow in the same media. Because *E.hisolytica* is the only species that cause invasive disease, differentiation from the closely related, more common, and morphologically identical species *E. dispar* in particular is desirable, in order to prevent unnecessary chemoprophylaxis. Until less laborious method become widely tested and implemented isoenzyme analysis will remain the standard for separation of these two parasites.

The most common source of material will be stool samples. In rare instances rectal biopsies or liver abscess aspirates have been the starting point for cultures. In the latter case since the abscess is sterile, addition of a bacterial flora is used for the direct establishment of monixeniccultures, unless the stool sample is several approaches to the establishment of cultures. Procedure for it first large beaker was taken then 1 lit distilled water was taken ,7 gm. of sodium chloride was added then heat and 15 gm. of Bacto TM agar media was added. Then again heat

after the heat the solution must be cool and taken into a small glass bottle by a syringe .Then placed in the autoclave, after completed the auto clave, the liquid solution bottle taken into a box and the box must be settled by an angle state because the angle bottle can be suitably for parasite.

2.6 Preparation of Rice Starch

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

Fig. 25: Incubator

The final concentration of diluted rice starch is 5 mg/ml. Before use, the rice was resuspended by vortexing or vigorous shaking and pippeted the desired volume into culture tubes with medium making sure that the stock rice stays in suspension. Different isolates require varying amounts of rice starch but 0.2 ml is often a suitable amount to add per culture tube. *Entamoeba* will not

ingest all forms of rice .Most important is the size of the rice particle as it must be within the ameba's ability to phagocytes it. [20]

2.7 Diphasic Media

Locke-egg (LE) Medium

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

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

After that when the solution is ready then autoclave it for 15 minutes at 121° C and at 15 lbs. pressure. It must be cooled to room temperature and removed any precipitate by filtration .And then re-autoclaved to sterilize. [50]

Preparation of Egg Slant

To prepare the egg slant the first procedure is surface sterilize fresh hens eggs by flaming in 70% ethanol and break into a graduated cylinder. Then add 12.5 ml Locke's solution per 45 ml of egg. After this emulsify in a waring-type blender and filter through gauze into a flask. Then it must be placed under the vacuum to draw out all air bubbles. Then the second stage is the addition of 5 ml amounts of the emulsified egg to standard 16x125 mm culture tubes and autoclave at 100° C for 10 minutes. The resulting egg slants should be free of bubbles. Cool to room temperature and overlay slants with 6 ml Locke's solution and autoclave for 15 minutes at 121° C and at 15 lbs. pressure. After cooling to room temperature tighten the caps and refrigerate for up to 6 months. [50]

Preparation of Robinson's Medium

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

0.5% Erythromycin: 0.5% Erythromycin is prepared in distilled water and filters then sterilizes and finally refrigerate.

20% Bactopeptone: 20% Bactopeptone is prepared in distilled water. Autoclave the solution and refrigerate.

10X Phthalate solution stock: It is prepared by mixing following ingredients:

102 g Potassium Hydrogen Phthalate

50 ml of 40% Sodium Hydroxide

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

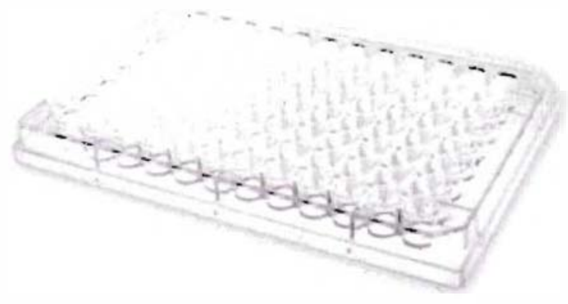


Fig. 26: ELISA Plate

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

- ✓ 25.0 g Sodium Chloride
- ✓ 10.0 g Citric Acid
- ✓ 2.5 g Potassium Phosphate, monobasic
- ✓ 5.0 g Ammonium Sulfate
- ✓ 0.25 g Magnesium Sulfate.$7H_2O$
- ✓ 20 ml 85% Lactic Acid solution.

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

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

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

Preparation of Agar Slants

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

2.8 Monophasic culture media

TYSGM-9

TYSGM-9 it is one kind of mono phasic media which is prepared by dissolving the following compounds. The compounds are:

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

To prepare the 950 ml solution with distilled water dispenses it in 95 ml amounts and adds 0.2 g bovine gastric mucin to each bottle. Then it autoclave for 15 minutes at 121° C with 15 lbs. pressure and store in the refrigerator. Before use, add 0.1 ml of a filter sterilized 5% stock of Tween 80 in distilled water and 5 ml of heat inactivated adult bovine serum. At last dispense in 8 ml amounts into 16x125mm culture tubes. [18]

East West University LIBRARY 43, Mohakhali, C/A

LYSGM

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

2.9 Axenic Culture Media

One constant problem facing those who are relies on axenic cultures is and fastidiousness of these organisms. Although the others are also affected to a significant degree, this is especially true of *E. histolytica*. 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, 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 tubescan cause. 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. [18]

Procedure for the Preparation of Axenic Culture Media for *E. histolytica*

TYI-S-33

To prepare TYI-S-33 dissolve the following in this orderin 600 ml of deionized or glass-distilled water, 1.0 g of potassium phosphate, dibasic, 0.6 g of potassium phosphate, monobasic, 2.0 g of sodium chloride, 20.0 g of casein digest peptone, 10.0g of yeast extract, 10.0 g of glucose, 1.0 g of L-cysteine hydrochloride, 0.2 g of ascorbic acid, and 1.0 ml of ferric ammonium citrate. [20]
The final volume was bought to 880 ml and pH to 6.8 using 1 N sodium hydroxide solution. Dispense in 88ml amounts into125ml glass bottles and autoclave for 15 min at 121°C under a pressure of 15 lbs. Then sterile TYI base can be stored frozen at 20°C for several months. Vitamin mix 18 unlike earlier mixtures used in the axenic culture of *E. histolytica*, contains only those vitamins known to be required by the parasite. It is also available commercially. [20]
(a) Step 1. The following four solutions must be prepared and then combine.(i) Dissolve 45 mg of niacinamide, 4 mg of pyridoxal hydrochloride,23 mg of calcium pantothenate, 5 mg of thiamine hydrochloride, and 1.2 mg of vitamin B_{12} in a final volume of 25 ml of water.(ii) Dissolve 7 mg of riboflavin in water using the minimum amount of 0.1 N sodium hydroxide,

bringing the final volume to 45 ml. (iii) Dissolve 5.5 mg of folic acid in water using the minimum amount of 0.1 N sodium hydroxide, bringing the final volume to 45 ml. (iv) Dissolve 2 mg of D-biotin in water bringing the final volume to 45 ml. If the combined solution is cloudy, it indicates that the pH is too high due to too much sodium hydroxide having been used in solutions ii and iii and the mixture must be discarded.

(b) Step 2. In step 2 dissolved 1 mg of DL-6-8-thioctic acid in 5 ml of 95% ethanol. Add 500 mg of Tween 80, bringing the volume to 30 ml with water. Combine the solutions from steps 1 and 2, bring the final volume to 200 ml with distilled water, and sterilize through a 0.22μm pore size filter. Store in 100 ml amounts at 4°C for up to 6 months. To complete TYI-S-33 medium for use in axenic cultivation add2.0 ml of vitamin mix 18 and 10 to 15 ml of heat-inactivated adult bovine serum to each 88 ml of TYI broth. In most cases 13 ml of TYI-S-33 per tube is the correct amount, as inocula for axenic *E. histolytica* are generally small in established cultures. The percentage of serum used varies among isolates but is usually either 10 or 15 %. [51]

YI-S

YI-S was developed as an alternative to TYI-S-33 due to difficulties in obtaining lots of casein digest peptone that would support adequate growth of *E. histolytica.* The recipe for YI-S is identical to that of TYI-S-33 except that casein digest peptone is replaced weight for weight by additional yeast extract, making the final concentration of yeast extract 3%.YI-S is not without its own problems, as the lot of yeast extract used is crucial to successful cultivation using this medium. [20]

LYI-S-2

In the course of developing YI-S, several combinations of liver digest and yeast extract are used. One of these designatedLYI-S-2, it is found to result in growth equal to that in TYI-S-33. Intent on producing a medium with as few biological ingredients as possible, the medium containing only yeast extract that is YI-S. It has been used in the long-term cultivation of several isolates of *E. histolytica* and a number of other *Entamoeba* species. [31]

2.10 Establishment of Cultures

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

Table 2: The quantities composition of LYI-S-media

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

2.11 General Considerations for the Preparation of Xenic Culture

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

The most common source of material will be stool samples and this is what is assumed below. In rare instances rectal biopsies or liver abscess aspirates have been the starting point for cultures. In the latter case, since the abscess is sterile, addition of a bacterial flora is necessary before inoculation of amoebae into xenic culture. Such material has also been used for the direct establishment of monoxenic cultures. Unless the stool sample is from a patient with dysentery the amoebae will be in the encysted form. This allows for several approaches to the establishment of cultures. [19]

2.12 Elimination of Unwanted Organisms

One of the banes of xenic cultivation is the likelihood of unwanted organisms outgrowing the desired ameba. The most frequent source of this problem is *Blastocystishominis* which may be the most common parasitic infection of humans. This organism is often missed on stool examination but grows luxuriantly in all the media used to cultivate xenic *Entamoeba.* Some authors control the growth of *B. hominis* with acriflavin but this also has an adverse effect on the bacterial flora and, directly or indirectly, on the ameba of interest. We have successfully used two methods to eliminate *B. hominis* from *Entamoeba* cultures

In first method, cysts are treated with 0.1N hydrochloric acid at room temperature for 10 minutes, washed thoroughly with distilled water, and re-inoculated into culture medium to which a suitable bacterial flora has been added. The acid kills the bacteria, any fungi *B. hominis,* intestinal *Trichomonas* and any non encysted amoebae while leaving the cysts intact and viable.

The bacterial flora used in the above method is separated from another xenic culture by inoculating into culture medium, without rice starch, a small amount of supernatant from an established culture, sub culturing 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 ameba's growth. The flora can be stored at 4°C indefinitely. [19]

The second method is called "Smedley method" and is used when *B. hominis* appears in cultures. It does not rely on cysts being present and so has advantages over the method of Dobell and Laidlaw in that respect. However, the method may need to be repeated a couple of times before the *B. hominis* is completely eliminated. Cultures are pelleted and the pellet, which contains a mixture of all the organisms present, is resuspended in distilled water at room temperature for 15 minutes. The material is then re pelleted and inoculated into fresh culture medium. Perhaps

surprisingly, many *Entamoeba* trophozoites survive this treatment while *B. hominis* generally does not. A few cells of *B. hominis* may survive and start to grow, and the procedure will then need to be repeated. The advantage of Smedley's method is its simplicity and the fact that no separate bacterial flora is needed. Other unwanted organisms such as fungi and *Trichomonas* will usually disappear from xenic cultures after several passages. [49]

2.13 Isolation

In our experience LE has proven to be the best medium for primary isolation of *Entamoeba* species from stool, although we have limited experience with Robinson's medium which is widely used for this purpose. TYSGM-9 can also be used for isolation but its primary utility is in generating large numbers of amoebae from established cultures. The numbers of amoebae obtained from the two diphasic media are generally low in comparison with TYSGM-9, but their success in primary isolation amoebae from microscopically positive stool is higher. In all cases, rice starch is added to the medium before inoculation, as are the antibiotics when needed.

Material for inoculation of xenic cultures can be prepared in several ways. Most commonly, stool samples are emulsified in saline and passed through a mesh to remove most of the larger particulates from the material before addition to the culture medium. It is always a good idea to include portions of the stool that appear bloody if these are present. Stool fractionation by flotation in zinc sulfate is also used as this reduces the amount of debris while concentrating the cysts present in the sample. We routinely use more than one medium, if available, and set up duplicate cultures where one has antibiotics added and the other not. Penicillin/streptomycin or erythromycin is the antibiotics of choice as they appear to have little direct effect on the amoebae. However, the widespread occurrence of antibiotic resistance in bacteria makes it impossible to generalize about the amount and type of antibiotics necessary to control the growth and rice-splitting activity of human bacterial flora.

Culture tubes, containing medium and rice starch, to which stool derived material has been added are incubated vertically at 35.5° C for 48 hours before examination. Ideally, examination should be done in situ if possible. This can be accomplished by slanting the tubes and using an inverted microscope. Amoebae can be observed adhering to the walls of the glass culture tubes above the fecal material. In situ examination is much easier in monophasic medium due to its relative clarity. Alternatively, a drop of sediment can be extracted from the tube for examination on a

microscope slide. If no growth is observed at 48 hours a blind passage should be made. The culture tube is chilled in an ice water bath for 5 minutes and most of the liquid overlay discarded to leave less than 1 ml in the tube. The sediment is resuspended in the remaining fluid and transferred to a fresh culture tube with medium and rice. After incubation for a further 48 hours the culture is re examined as above. If no amoebae are seen the subculture method is identical except that the size of the inoculum will be gradually reduced as the numbers of amoebae increase. Initially, it is usually helpful to centrifuge the cultures and split the pellet among the recipient tubes. This can be done by chilling the culture tube, inverting several times to detach the adherent amoebae, and transferring the liquid phase to an empty culture tube before pelleting. [17]

2.14 Axenization

Axenization of *E. histolytica* is along and laborious procedure involving gradual adaptation of the parasite to a new way of life. The medium can be a specialized monoxenic culture medium but we have also use success initiating such cultures using one of the axenic media such as TYI-S-33. The monoxenic associate we have used most frequently is *C. fasciculata* .This insect flagellate is grown as a stock culture at room temperature and added to the monoxenic culture of amoebae at each subculture, as *Crithidia* does not grow at the incubation temperature of the amoebae; the amount added varies. *T. cruzi* Culbertson has also been used successfully as the associated organism but is not recommended due to the potential for infection, even though this strain is of very low virulence. The antibiotics added vary both in type and amount depending on the sensitivities of the flora in which the amoebae were growing. We have used a cocktail of rifampin, amikacin, oxytetracycline, and cefotaxime with good success. Except for the first agent, these have little effect on the ameba. 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. [17]

At least two subcultures in the absence of antibiotics should be performed before the cultures can be considered free of bacteria. This can be verified using standard aerobic and anaerobic testing

procedures for bacteria, including mycoplasmas and fungi. Established monoxenic cultures, those in which growth is reproducible and bacteria are absent, are then used to initiate axenic cultures. This uses the same medium but with no *Crithidia* added. After a few subcultures the flagellates disappear as a result of dilution and ingestion. It is often helpful, although not always necessary to add a small amount of Noble agar to the tubes. It appears to form a substrate for the amoebae. In addition, the tubes should be incubated vertically rather than at 5° to the horizontal. Often the culture will flourish initially and then numbers will crash. It is the crisis point that the cultures are most vulnerable. As long as a few live cells persist, it is worth continuing to replace the medium every few days. With luck, the numbers will gradually start to increase again, and eventually addition of the agar will no longer be needed. When established, the axenic cultures can be incubated at 5° to the horizontal. [20]

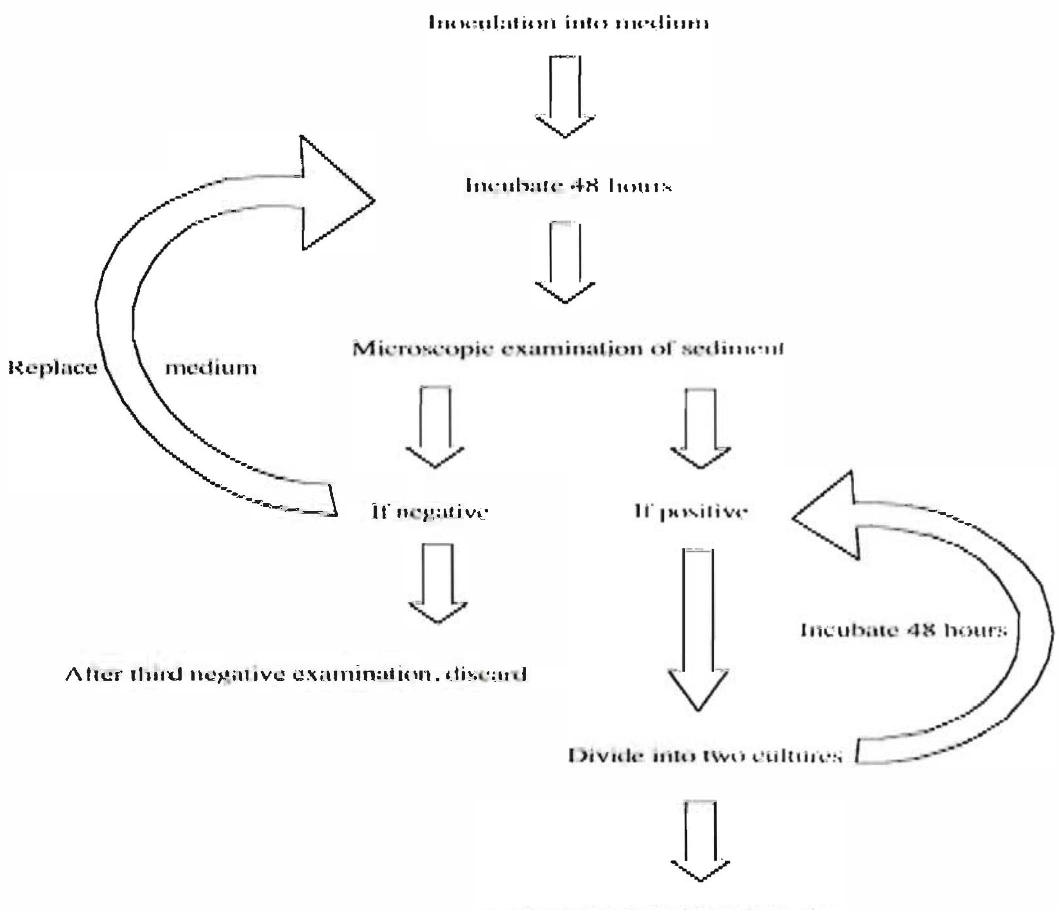


Fig. 27: Flow diagrams illustrating the stages in establishing luminal protists in culture

2.15 Principles of Inducing Encystment of *E. histolytica*

The methods of inducing encystment of *E. histolytica* are based on Dobell and Laidlaw's discovery that cyst-production may sometimes be temporarily increased by cultivating the amoebae in starch-free media for one or two generations, and then transferring them to media containing this substance but the results are uncertain, and the number of cysts produced in any culture cannot be predicted.

Here at present we use a protocol that is used for many years in the NIH Laboratory of Parasitic Diseases. Three things are of special concern in obtaining cysts they are the media, bacterial flora, and rice starch. Some media are better than others for this purpose. LE medium is the one with which we and others have had excellent results

The accompanying bacterial flora presents in a xenic culture plays an important role in the process of encystment. It is good practice for those requiring steady source of large numbers of cysts to isolate and maintain the bacterial flora of a xenic culture in which cysts regularly form spontaneously. [19]

Protocol of Encystment

(a) Day 1: At first the process is begin with three amoebae rich 48 h cultures in LE medium. Harvest them by chilling the culture tubes for5 min in an ice water bath then invert the tubes 10 times to mix contents and free amoebae adhering to the glass and egg slant, and centrifuge for 3 min at 275 x *g*. Remove and discard all but 1 ml of the spent overlay. Finally resuspend pelleted amoebae, pool, and transfer equal amounts to six tubes of LE medium without rice. Incubate the cultures in an upright position for 72 h.

(b) Day 4: Harvest each of the six cultures: Chill, remove and discard all but 1 ml of overlay. Mix remaining overlay of each culture and transfer equal amounts to two tubes of LE medium without rice. There will now be 12 cultures. Then it is incubate for 48 h.

(c) Day 6: Harvest the 12 cultures and subculture as on day 4 and then the cultures must incubate for 48 h.

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

(e) Day 10: Remove a small drop of sediment from each culture, stain with Lugol's iodine solution, and search for presence of quadrinucleate cysts. If found, harvest cultures as on day1. Remove overlay, leaving only the sediment. Pool sediments and wash two times with distilled water. Cysts will remain viable from 10 to 14 days when stored at 4°C. If cysts are not found, then incubate it with additional 24 h.

Protocol of Excystment of Cysts Induced in vitro

Inducing *E. histolytica* to excyst is relatively easy compared to getting the ameba to encyst. If the goal is to propagate the amoebae in a xenic environment, then the medium in which the cysts were induced is used, in this case LE medium. If the goal is to excyst them in a bacteria-free environment, any of the monophasic liquid media devised for axenic culture can be used. In the latter case freshly prepared medium must be used. Best results are obtained when at least 50% of the cysts produced are in the quadrinucleate stage. Usually no more than 25% of the cysts can be expected to excyst.

To induce excystment, the cysts are first treated to remove unwanted organisms. They are then placed in a tube of LE medium inoculated with a suitable bacterial flora for xenic growth or in a medium capable of sustaining axenic growth. Upon incubation most of the cysts capable of undergoing excystation.

2.16 Rexenization of Axenically Cultivated *E. histolytica*

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

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

(ii) Chilling a 72-h culture of axenically cultivated amoebae in an ice-water bath for 5 min. Invert culture tube 10 times to dislodge amoebae from glass surfaces. Centrifuge 3 min at 275x *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 1×10^5,2×10^5, and 4×10^5 amoebae, respectively.

(iv) Incubate 48 h. Remove all but approximately 1 ml of overlay. Resuspend the sediment located at the interface of the slant and overlay. Examine a drop with a microscope. The majority of inoculated amoebae will have died. Select the best of the three cultures and subculture.
(v) The number of amoebae transferred can be determined only by trial and error. In the early stages of establishing the culture, transfer one-half of the material from the old culture to each of two tubes of fresh medium. Later, as amebic growth improves, transfer smaller portions, e.g., one-third to one fourth.

2.17 Maintenance of Culture

When the culture established all parasites then they are handled in the same way. Xenic cultures of *E. histolytica* is routinely passaged at 48 to 72h intervals, usually a Sunday- Tuesday schedule is convenient. Occasionally cultures of these organisms will be found that do better with twice weekly subculture. The inoculum size for the longer incubation period should be smaller than that for shorter incubations. However, variation among isolates and flora means that no generalities can be made regarding the size of inocula or the amount of rice and antibiotics to be added to the medium for optimal growth. It is very much a case of trial and error combined with experience in evaluating growth of cultures that leads to successful establishment of these parasites in xenic culture. It is recommended that xenic cultures be passaged using two or more inoculum sizes to ensure a successful subculture. A significant threshold effect can sometimes be encountered, in which a certain inoculum size gives rise to a healthy culture but an inoculum smaller by as little as 50 μl may result in no growth. An increased inoculum volume may be warranted for the subsequent subculture to compensate for the dead amebae. The method for subculturing is essential. Cultures are chilled in an ice water bath for 5 minor 10 min to release trophozoites attached to the glass culture tube. Tubes are inverted several times to disperse the cells and a measured inoculum is passed aseptically to a culture tube that contains the fresh medium. The tubes are capped tightly and incubated at 36 to 37°C.

2.18 Preparation of Antimicrobial Agent

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

2.19 *In vitro* Drug Sensitivity Assay

Drug sensitivity assay of the sample was carried out by using microtiter plates. In row A 200 micro liter of the standard was given and the samples were given. In all other rows (B-H) the 100 micro liter medium was added and dilution of the drugs were performed down the plate then mixed properly. 100 micro liter of the medium from the last row (H) was discarded to maintain the equality of the concentration of the drugs. The final concentration of the drug was 0.07, 0.14, 0.29, 0.58, 1.15, 2.3, 4.6 and 9.2 μM. Further 100 μL of parasite suspension was added to all the rows (A-H). Each test included the control where no drug is present. Then plastic strip was used to cover the plate. Plates were incubated at 37°C and examined after 1 or 2 h under a microscope to check for the presence of amoeba. After 4 h the plate was taken from the incubator. Then the viable parasites were counted by haemocytometer under microscope in each of the rows.

East West University
EAST WEST
LIBRARY
43, Mohakhali, C/A

Result

3. Result

3.1 Measurement of Amebicidal Activity

According to the study, Robic and Ornil drug have a good antiamebic activity. The clinical isolates of *Entamoeba histolytica* were treated with these two brands at different concentration. The experimental concentrations of this experiment were 0.14, 0.29, 0.58, 1.15, 2.3 and 4.6. 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 Robic and Ornil 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 nonviable counts of *Entamoeba histolytica* were counted and recorded in a table and in a table which demonstrated that Ornidazole having good sensitivity against clinical isolates of *Entamoeba histolytica.*

3.1.1 Viable count of *Entamoeba histolytica* after 24 hours incubation (Standard and Ornil)

When the preparation was completed then it was incubated in 24 hours with different concentration of standard and ornil for Ornidazole.

The initial counts of the parasite were $1.575 \times 10^5 mL^{-1}$ and the initial count of the control media were $3.95\times10^5 mL^{-1}$for standard and ornil. After 24 hours the viable count of *Entamoeba histolytica* were $0.4\times10^5 mL^{-1}$, $0.3.75\times10^5 mL^{-1}$, $0.25\times10^5 mL^{-1}$, $0.20\times10^5 mL^{-1}$ and $0.15\times10^5 mL^{-1}$ when the concentration were 0.14, 0.29, 0.58, 1.15 and 2.3 μM respectively. The viable count of *Entamoeba histolytica* was decreased to $0.12\times10^5 mL^{-1}$ when the concentration of standard was increased to 4.6μM.

On the other hand, after 24 hours the viable count of *Entamoeba histolytica* were 1.32×10^5 mL^{-1} , 1.2×10^5 mL^{-1} , 1.15×10^5 mL-1, 1.02×10^5 mL^{-1} and 0.65×10^5 mL--1 when the concentration were 0.14, 0.29, 0.58, 1.15 and 2.3 μM respectively. The viable count of Entamoeba histolytica was decreased to 0.45×10^5 mL^{-1} when the concentration of ornil was increased to 4.6 μM.

The numbers of parasites are increased in control which 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 are Ornidazole decreased.

Table 3: Viable counts of *Entamoeba histolytica* after 24 hours incubation (Standard and Ornil)

Concentration of Ornidazole (µM) Standard	Viable count of *E. histolytica*	Concentration of Ornidazole (µM) Drug(Ornil)	Viable count *E. histolytica*
4.6	0.12×10^5 mL^{-1}	4.6	0.45×10^5 mL^{-1}
2.3	$0{\cdot}15\times10^5$mL^{-1}	2.3	0.65×10^5mL^{-1}
1.15	0.20×10^5 mL^{-1}	1.15	1.02×10^5 mL^{-1}
0.58	0.25×10^5mL^{-1}	0.58	1.15×10^5mL^{-1}
0.29	0.3×10^5mL^{-1}	0.29	1.2×10^5mL^{-1}
0.14	0.4×10^5mL^{-1}	0.14	1.32×10^5mL^{-1}

Table 4: Percentage of viable *Entamoeba histolytica* after 24 hours incubation (Standard and Ornil)

Concentration of Ornidazole (µM) Standard	Viable count of *E. histolytica in* percentage	Viable count of *E. histolytica in* percentage (Ornil)
4.6	7.61 %	28.66%
2.3	9.52 %	41.40%
1.15	12.69 %	64.96%
0.575	15.87 %	73.24%
0.288	19.04 %	76.43%
0.144	25.35%	84.07%

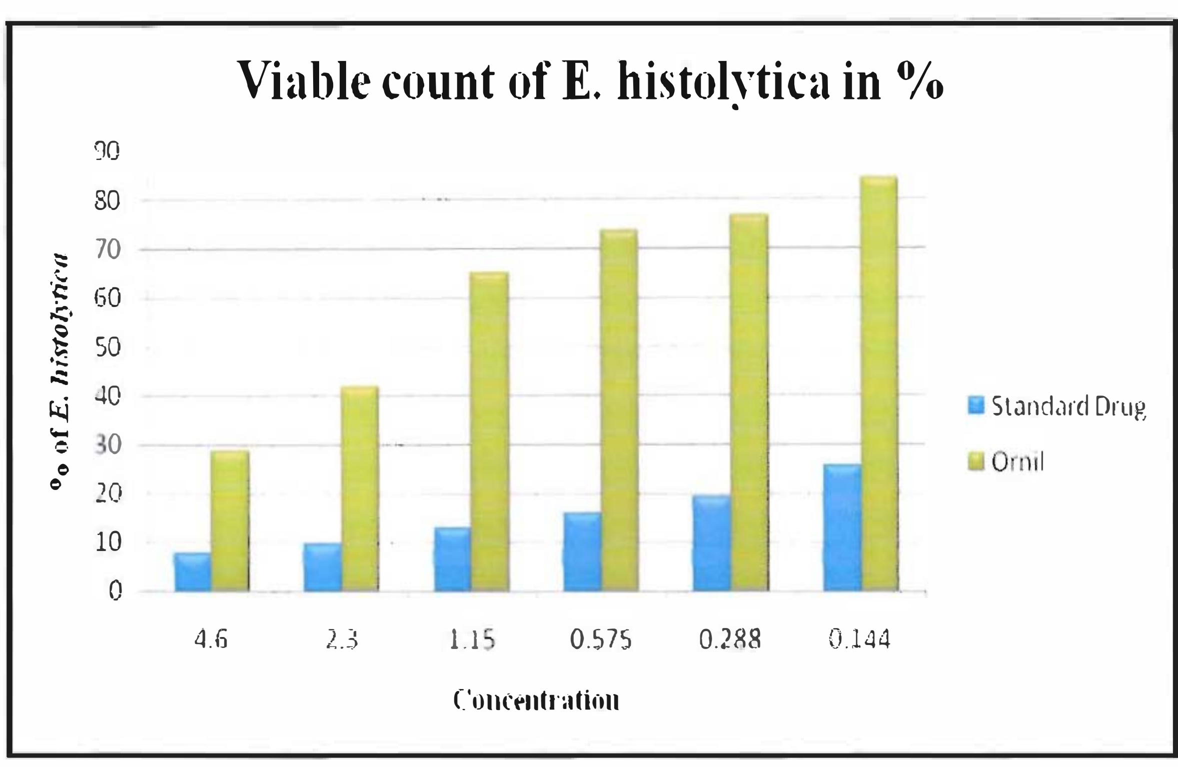


Fig. 28: Percentages of the viable counts *Entamoeba histolytica* after 24 hours incubation (Standard and Ornil)

3.1.2 Non viable count of *Entamoeba histolytica* after 24 hours incubation (Standard and Ornil)

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

The initial count of the parasite were $1.575 \times 10^5 mL^{-1}$ and the initial count of the control media were $3.95 \times 10^5 mL^{-1}$. After 24 hours the nonviable count of *Entamoeba histolytica* were $1.17 \times 10^5 mL^{-1}$, $1.25 \times 10^5 mL^{-1}$, $1.1.32 \times 10^5 mL^{-1}$, $1.37 \times 10^5 mL^{-1}$ and $1.42 \times 10^5 mL^{1}$ when the concentration were 0.14, 0.29, 0.58, 1.15 and 2.3 μM respectively. The nonviable count of *Entamoeba histolytica* was increased to $1.45 \times 10^5 mL^{-1}$ when the concentration of Ornidazole was increased to 4.6 μM for standard drug.

Table 5: Non viable counts of *Entamoeba histolytica* after 24 hours incubation (Standard and Ornil)

Concentration of Ornidazole (µM) Standard	Non Viable count of *E. histolytica*	Concentration of Ornidazole (µM) Standard	Non Viable count of *E. histolytica(ornil)*
4.6	1.45×10^5 mL^{-1}	4.6	1.12×10^5 mL^{-1}
2.3	1.42×10^5mL^{-1}	2.3	0.92×10^5 mL^{-1}
1.15	1.37×10^5 mL^{-1}	1.15	0.55×10^5 mL^{-1}
0.575	1.325×10^5mL^{-1}	0.575	0.42×10^5 mL^{-1}
0.288	1.25×10^5mL^{-1}	0.288	0.37×10^5 mL^{-1}
0.144	1.17×10^5mL^{-1}	0.144	0.25×10^5 mL^{-1}

Table 6: Percentage of non viable *Entamoeba histolytica* after 24 hours incubation (Standard and Ornil)

Concentration of Ornidazole (µM) Standard	Non viable count of *E. histolytica in* percentage	Non viable count of *E. histolytica in* percentage (Ornil)
4.6	92.06 %	71.34%
2.3	90.15 %	58.6%
1.15	87.26 %	35.05%
0.575	84.16 %	26.76%
0.288	79.36 %	23.57%
0.144	74.16%	15.93%

On the other hand, after 24 hours the non viable count of *Entamoeba histolytica* were 0.25×10^5 mL^{-1}, 0.37×10^5 mL^{-1}, 0.42×10^5 mL^{-1}, 0.55×10^5 mL^{-1} and 0.92×10^5 mL^{-1} when the concentration

were 0.14, 0.29, 0.58, 1.15 and 2.3 μM respectively. The nonviable count of *Entamoeba histolytica* was increased to 1.12×10^5 $mL^{-1}$when the concentration of Ornidazole was increased to 4.6 μM for ornil drug.

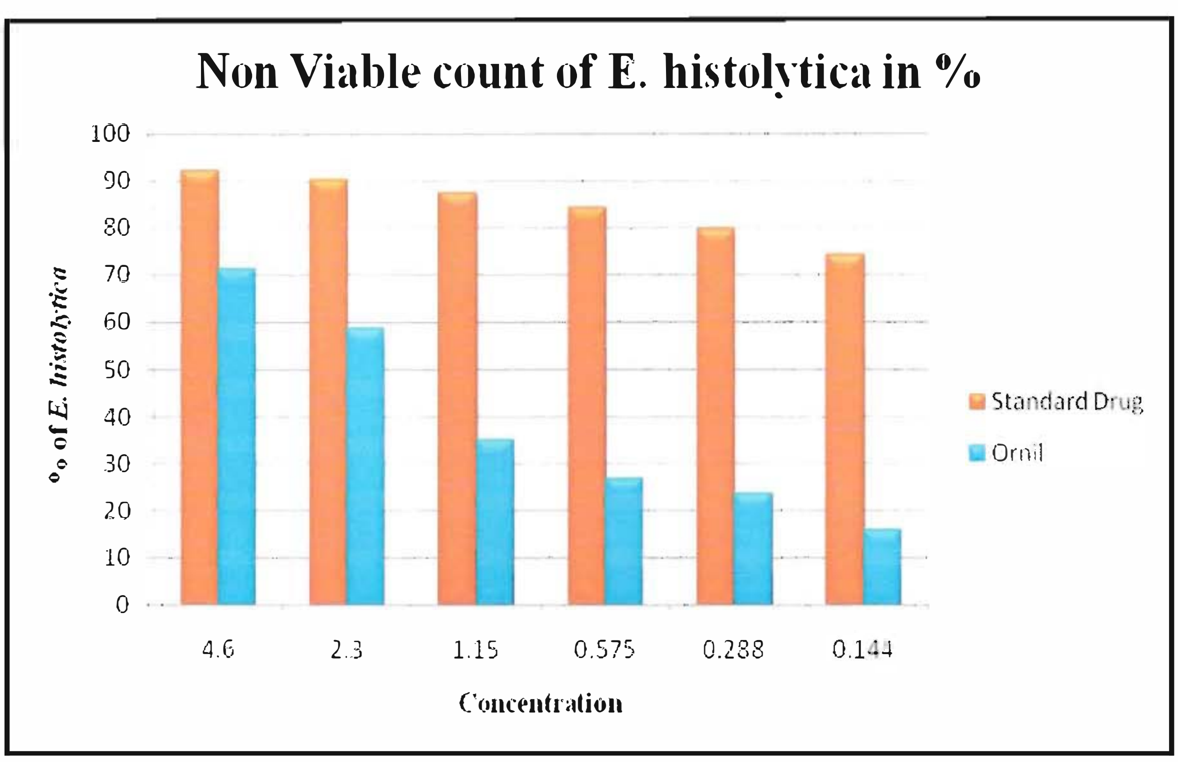


Fig. 29: Percentages of the non viable counts *Entamoeba histolytica* after 24 hours incubation (Standard and Ornil)

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 nonviable count are increased when the concentrations of Ornidazole are increased.

From the result it can be concluded that when the concentration is 4.6 μM then viable count is 7.61% and 28.66% also the nonviable count is 92.06% and 71.34% for standard and ornil drug On the other hand when the concentration is 0.144 μM the viable count is 25.35% and 84.07% also the nonviable count is 74.26% and 15.93% for standard and ornil drug. So when the concentrations of Ornidazole are increased then the viable counts of *Entamoeba histolytica* are decreased and ultimately the nonviable count of *Entamoeba histolytica* increased when the

concentrations of the drug are increased. It shows that Ornidazole having good antiamebic activity.

3.1.3 Viable count of *Entamoeba histolytica* after 24 hours incubation (Standard and Robic)

The initial count of the parasite were 1.575 ×105 mL^{-1} and the initial count of the control media were 1.8× 10^{5} mL^{-1} for standard and Robic drug.

Table 7: Viable counts of *Entamoeba histolytica* after 24 hours incubation (Standard and Robic)

Concentration of Robic (μM) Standard	Viable count of *E. histolytica*	Concentration of Ornidazole (μM) Drug (Robic)	Viable count *E. histolytica*
4.6	0.22 ×10^{5} mL^{-1}	4.6	0.17×10^{5} mL^{-1}
2.3	0.47×10^{5} mL^{-1}	2.3	0.22×10^{5} mL^{-1}
1.15	0.57×10^{5} mL^{-1}	1.15	0.25×10^{5} mL^{-1}
0.575	0.67×10^{5} mL^{-1}	0.575	0.32×10^{5} mL^{-1}
0.288	0.95×10^{5} mL^{-1}	0.288	0.37×10^{5} mL^{-1}
0.144	1.0×10^{5} mL^{-1}	0.144	0.42×10^{5} mL^{-1}

This figure shows that the viable count of *Entamoeba histolytica* after treating with different concentration of Ornidazole for 24 hours. The initial count of *Entamoeba histolytica* is 1.57×10^{5}mL^{-1}.After 24 hours the viable count of *Entamoeba histolytica* were 1.0×10^{5}mL^{-1},0.95×10^{5} mL^{-1},0.67×10^{5}mL^{-1}, 0.57×10^{5}mL^{-1}, 0.47×10^{5}mL^{-1} and 0.22×10^{5}mL^{-1} when the concentration were 0.14, 0.29, 0.58, 1.15, 2.3 and 4.6 μM for standard drug.

On the other hand, After 24 hours the viable count of *Entamoeba histolytica* were 0.42×10^{5} mL^{-1}, 0.37×10^{5} mL^{-1}, 0.32×10^{5} mL^{-1}, 0.25×10^{5} mL^{-1}, 0.22×10^{5} mL^{-1} and 0.17×10^{5} mL^{-1} when the concentration were 0.14, 0.29, 0.58, 1.15, 2.3 and 4.6 μM for Robic drug.

Table 8: Percentage of viable *Entamoeba histolytica* after 24 hours incubation (Standard and Robic)

Concentration of Ornidazole (µM) Standard	Viable count of *E. histolytica in* percentage	Viable count of *E. histolytica in* percentage (Robic)
4.6	14.01 %	10.82%
2.3	29.29 %	14.01%
1.15	36.30 %	15.92%
0.575	42.67 %	20.38%
0.288	60.50 %	23.56%
0.144	63.69 %	26.75%

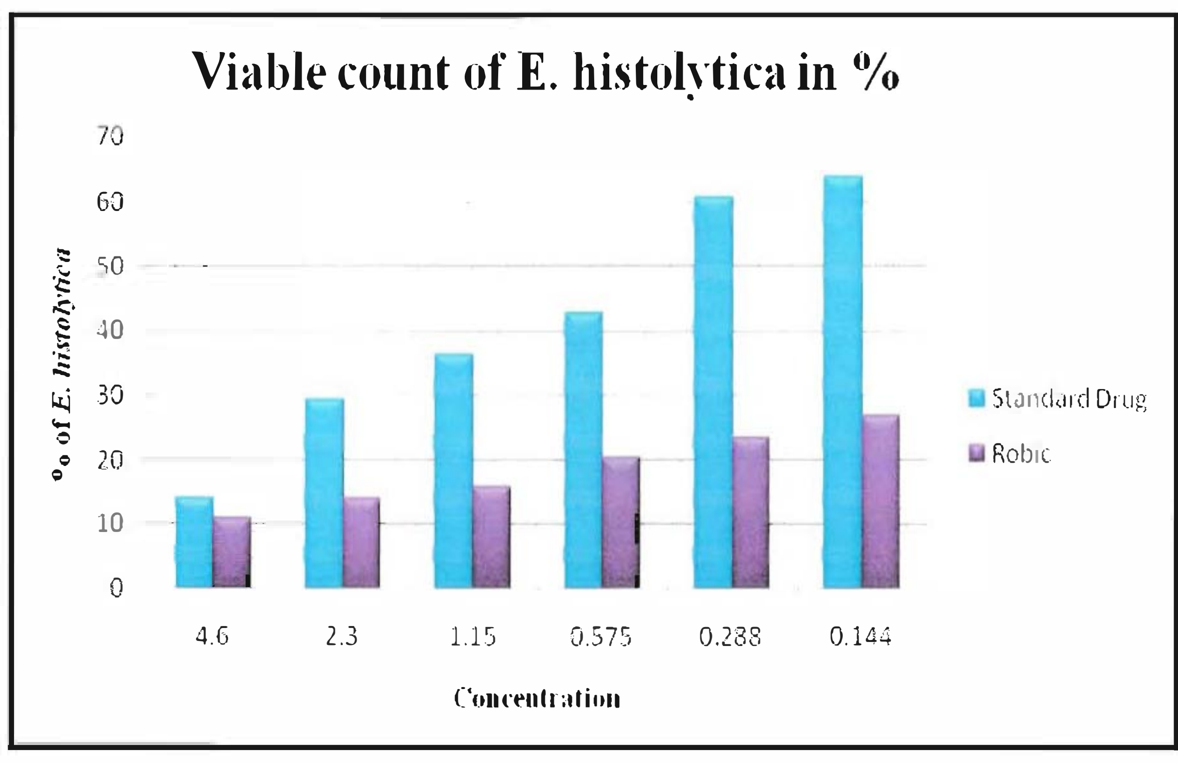


Fig. 30: Percentages of the viable counts *Entamoeba histolytica* after 24 hours incubation (Standard and Robic)

3.1.4 Non viable count of *Entamoeba histolytica* after 24 hours incubation (Standard and Robic)

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

Table 9: Non viable counts of *Entamoeba histolytica* after 24 hours incubation (Standard and Robic)

Concentration of Ornidazole (µM) Standard	Non Viable count of *E. histolytica*	Concentration of Ornidazole (µM) Drug (Robic)	Non Viable count of *E. histolytica(Robic)*
4.6	1.35×10^5 mL^{-1}	4.6	1.40×10^5 mL^{-1}
2.3	1.10×10^5 mL^{-1}	2.3	1.35×10^5 mL^{-1}
1.15	1.0×10^5 mL^{-1}	1.15	1.32×10^5 mL^{-1}
0.575	0.9×10^5 mL^{-1}	0.575	1.25×10^5 mL^{-1}
0.288	0.62×10^5 mL^{-1}	0.288	1.20×10^5 mL^{-1}
0.144	0.57×10^5 mL^{-1}	0.144	1.15×10^5 mL^{-1}

The initial count of the parasite were $1.57 \times 10^5 mL^{-1}$and the initial count of the control media were $1.8\times 10^5 mL^{-1}$ .After 24 hours the nonviable count of *Entamoeba histolytica* were $0.57\times 10^5 mL^{-1}$,$0.62\times 10^5 maL^{-1}$,$0.9\times 10^5 mL^{-1}$,$1.0\times 10^5 mL^{-1}$,$1.10\times 10^5 mL^{-1}$ and $1.35\times 10^5 mL^{-1}$when the concentration were 0.14, 0.29, 0.58, 1.15, 2.3 and 4.6 µM for standard drug.

On the other hand, After 24 hours the nonviable count of *Entamoeba histolytica* were 1.15×10^5 mL^{-1}, 1.20×10^5 mL^{-1}, 1.25×10^5 mL^{-1}, 1.32×10^5 mL^{-1}, 1.35×10^5 mL^{-1}, and 1.40×10^5 mL^{-1}, when the concentration were 0.14, 0.29, 0.58, 1.15, 2.3 and 4.6 µM respectively for Robic drug.

The numbers of parasites are increased in the control where it indicates that the numbers of parasite are increased after 24 hours incubation. So, the percentages of the nonviable count are increased when the concentrations of Ornidazole are increased.

Table 10: Percentage of non viable *Entamoeba histolytica* after 24 hours incubation (Standard and Robic)

Concentration of Ornidazole (µM) Standard	Non Viable count of *E. histolytica* *in* percentage	Non viable count of *E. histolytica in* percentage (Robic)
4.6	85.98 %	89.18%
2.3	70.06 %	85.99%
1.15	63.69 %	84.08%
0.575	57.32 %	79.62%
0.288	39.49 %	76.44%
0.144	36.30 %	73.25%

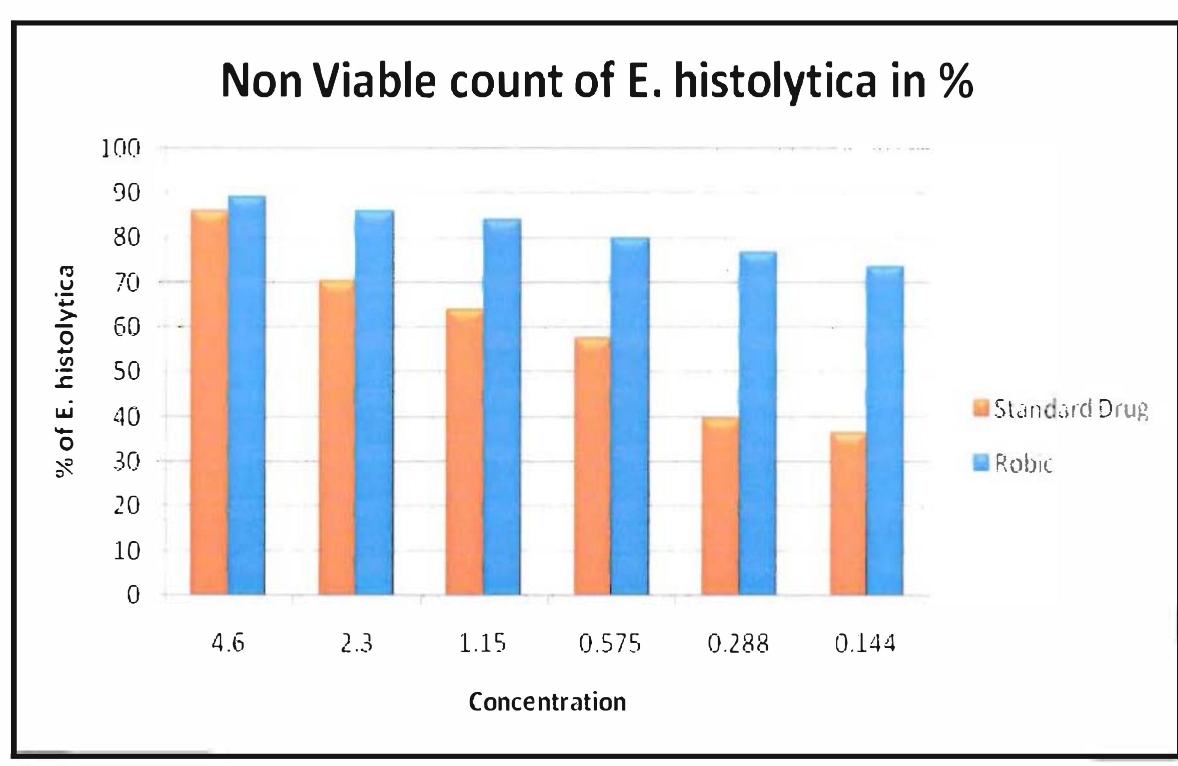


Fig. 31: Percentages of the non viable counts *Entamoeba histolytica* after 24 hours incubation (Standard and Robic)

From the result it can be concluded that when the concentration is 0.144 μM the viable count is 63.69% and 26.75% for standard and Robic drug. Nonviable count is 36.30% and 73.25% for standard and drug in same concentration. So, when the concentrations of Ornidazole are increased then the viable counts of *Entamoeba histolytica* are decreased and ultimately the nonviable count of *Entamoeba histolytica* increased when the concentrations of the drug are increased. It show that Ornidazole having antiamebic activity.

East West University LIBRARY 43, Mohakhali, C/A

Discussion

4. Discussion

The ultimate objective of this study is to determine the therapeutic efficacy of two brand of Ornidazole (Ornil and Robic) that is used against *Entamoeba histolytica* associated with amebiasis treatment.

Ornidazole, a 5- nitroimidazole derivative, is effective in the treatment of a broad range of parasitic infections. *In vitro*, it is active against several protozoa, including *T. vaginal* is, *E. histolytica, G. lamblia* and certain anaerobic bacteria. Ornidazole is a first-line choice for the treatment of illness caused by *Entamoeba histolytica* and *Giardia intestinalis* infection in immune competent adults and children. It is an option to be considered in the treatment of illness caused by other protozoa and helminthes. It is used for the treatment of amebiasis and all intestinal infections due to *Entamoeba histolytica* including amebic dysentery.

The clinical isolates of *Entamoeba histolytica* were treated with Ornil and Robic at different concentration. The experimental concentrations were 0.14, 0.29, 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 Ornil, Robic 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 Ornil and Robic having good sensitivity against clinical isolates of *Entamoeba histolytica.*

When the initial count of *Entamoeba histolytica* is 1.575×105 mL-1 then after 24 hours the viable count was 7.61% and 28.66% also the nonviable count is 92.06% and 71.34% for standard and ornil drug when the concentration was 4.6 μM.

On the other hand when the concentration is 0.144 μM the viable count was 25.35% and 84.07% also the nonviable count is 74.26% and 15.93% for standard and ornil drug.

The others viable count were0.4×105mL-1 , 0.3.75×105mL-1 , 0.25×105mL-1, 0.20×105mL-1 ,0.15×105mL-1 and 0.12×105mL-1 when the concentration were 0.14, 0.29, 0.58, 1.15, 2.3 and 4.6 μM for standard and 1.32×105 mL-1 , 1.2×105 mL-1 , 1.15×105 mL-1, 1.02×105 mL-1 and 0.65×105 mL-1 and 0.45×105 mL-1 for ornil drug.

According to this, after 24 hours the non viable count of *E. histolytica* were 1.17×105mL-1, 1.25×105mL-1, 1.1.32×105mL-1, 1.37×105mL-1 , 1.42×105mL1 and 1.45×105mL-1 when the concentration were 0.14, 0.29, 0.58, 1.15, 2.3 and 4.6 μM for standard drug.

And after 24 hours the nonviable count of *Entamoeba histolytica* were 0.25×105 mL-1, 0.37×105 mL-1, 0.42×105 mL-1, 0.55×105 mL-1 , 0.92×105 mL-1 and 1.12×105 mL-1 when the concentration were 0.14, 0.29, 0.58, 1.15 and 2.3, 4.6 μM for ornil drug.

When the initial count of *Entamoeba histolytica* was 1.575×105 mL-1 then after 24 hours the viable count were14.01 %and 10.82% also the nonviable count is 85.98 % and89.18%when the concentration was 4.6 μM for standard and Robic drug.

After 24 hours the when the concentration is 0.144 μM the viable count is 63.69% and 26.75% and Nonviable count is 36.30% and 73.25% for standard and Robic drug.

So, the percentages of the non viable counts are increased when the concentration of Ornil and Robic 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 9.2 μM concentration of these two brands. To compare with the previous study [52], it has found that the result of inhibition of *Entamoeba histolytica* is similar because about 100% inhibition of growth of *Entamoeba histolytica* was occurred when the concentration of Ornil and Robic is more than 4.6 μM. Robic drug is more sensitive than Ornil drug.

So, it is effective in the treatment of a broad range of parasitic infections. *In vitro* sensitivity study of Ornil and Robic against *Entamoeba histolytica* is high and it is an innovative treatment option against amebiasis.

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

Conclusion

5. Conclusion

In conclusion, it appears that two brands of Ornidazole are active against protozoa and anaerobic bacteria. Ornil and Robic have been used successfully in patients with vaginal trichomoniasis and amebiasis. These two brands of Ornidazole have few and mild side-effects, require a short one to three days course of treatment and are inexpensive. These two drugs can be given in different dosage forms. These drugs are administered orally, vaginally, or intravenously. These drugs are effective and relatively safe.

References

References

1. Amebiasis, Journal of Boston Public Health Commission-Communicable Disease Control Division. October 2002, USA
2. Amebiasis, Journal of County of San Diego-Health and Human Services Agency-Community Epidemiology, April 2005
3. Amebiasis, Journal of Communicable Disease Management Protocol: Manitoba Health public Health, November 2001
4. Amoebiasis, Retrieved January 05, 2011 from: http://www/veria.com/conditions-a-z/amoebiasis.html
5. Bansal, D., Malla, N., and Mahajan, R.C., Drug resistance in amoebiasis, Journal of Indian J Med Res, February 2006, 123: 115-118
6. Block, J. H. and Beale (Jr.), J. M. (2004), Wilson and Gisvold's Textbook of Organic Medicinal and Pharmaceutical Chemistry, 11th Ed, Philadelphia, Lippincott Williams & Wilkins
7. Bennett, C.E., Baxendine, A & Arcari, M., Diagnosing Medical Parasites Through Coprological Techniques, 2000.
8. Boeck, W. C., and J. Drbohlav., The cultivation of *Entamoeba histolytica*, 1925, 5:371-407.
9. Balamuth, W., Improved egg yolk medium for cultivation of *Entamoeba histolytica* and other intestinal protozoa. Am. J. Clin. Pathol, 1946, 16:380-384.
10. Calore, E.E., Calore, N.M.P & Cavaliere, M.J, Salpingitis Due to *Entamoeba histolytica*, Journal of The Brazilian Journal of Infectious Diseases, 2002, 6(2): 97-99
11. Causes of Amoebiasis, Retrieved January 09, 2011 from: http://www.skin-diseases.org/diseases/amoebiasis.html
12. Chua, W.P.S., Amebiasis, Journal of Heart to Heart Talk, April 2004
13. Cleveland, L. R., and E. P. Sanders., 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, 1930, 77:149-151.
14. Clark, C. G. and L. S. Diamond., Methods for cultivation of luminal parasitic protists of clinical importance, 2002, 15: 329-341
15. Dhawan, V. K, and Naparst, T. R, Amoebiasis: Treatment and Medication, Medscape, July 2010, USA

16. Dougherty, E. C., Journal of Introduction to axenic cultivation of invertebrate metazoa.., 1959, 77: 27-54.
17. Diamond, L. S., Journal of Techniques of axenic cultivation of *Entamoeba histolytica* Schaudinn, and *E. histolytica*-like amebae. J. Parasitol, 1968, 54:1047-1056.
18. Diamond, L. S., Journal of A new liquid medium for xenic cultivation of *Entamoeba histolytica* and other lumen dwelling protozoa., 1982, 68:958-959.
19. Dobell, C., and Laidlaw, P. P., Journal of Cultivation of *Entamoeba histolytica*., 1926, 18:283-318
20. Diamond, L. S., Journal of *In vitro* cultivation of protozoan parasites, 1983, 67-109
21. Fotedar, R., Stark, D., Beebe, N., Marriott, D., Ellis, J., and Harkness, J., Laboratory Diagnostic Techniques for Entamoeba Species, Journal of Clinical Microbiology reviews, July 2007, 20 (3): 511–532
22. Gebertsadik, A., Kebede, A., Mezemer, M., and Tasew, G., Detection and differentiation of two morphologically identical species of Entamoeba, Journal of Ethiop.J.Health Dev, 2004, 18(2):121-124
23. Global Research-Bangladesh-Summary of Past and Present NIAID Funded Research, National Institute of Allergy and Infectious Diseases, August 2010, USA
24. Gonzales, M.L.M., Dans, L.F., & Martinez, E.G., Antiamoebic drugs for treating amoebic colitis, Journal of Cochrane Database of Systematic Reviews, 2009, 2: 1-133
25. Gillin, F. D., and L. S. Diamond., Journal of Clonal growth of *Entamoeba histolytica* and other species of *Entamoeba* in agar. J. Protozoal, 1978, 25:539-543.
26. Halsey, J.L., Journal of Current approaches to the treatment of gastrointestinal infections: Focus on Nitazoxanide, Journal of Clinical Medicine: Therapeutics, 2009, 1:263–275
27. Hardman, J. G., Limbird, L. E. and Gilman, A. G. (2006), Goodman & Gilman's The Pharmacological Basis of Therapeutics, 11th Ed, New York, McGraw-Hill Companies, Inc.
28. Harvey, R. A. and Champe, P. C, Lippincott's Illustrated Reviews Pharmacology, 4th Ed, USA, Lippincott Williams & Wilkins
29. Hizarcioglu, S.Y., Özyazici, M & Ay, Z., Bioavailability File: Ornidazole, Journal of FABAD J. Pharm. Sci., 2004, 29:133-144
30. Haque, R., Hughes, M., Houpt, E., and Petri, W.A., Amebiasis, The New England Journal of Medicine, 2003, (348): 1565-1573.

31. Ho, L. C., M. Singh, G. Suresh, G. C., and Yap, E. H, Journal of Axenic culture of *Blastocystis hominis* in Iscove's modified Dulbecco's medium. Parasitol, 1993, 79:614-616s
32. Juniper, K., Amebiasis, Journal of Phil J Microbiol Infect Dis 1983, 1-11
33. Katzung, B. G. (2003), Basic and Clinical Pharmacology, 9th Ed, New York, McGraw-Hill Companies, Inc.
34. Kebede, A., Kassa, E., Ashenafi, S., Woldemichael, T., Polderman, A.M., and Petros, B., Amoebic liver abscess: A 20-year retrospective analysis at Tikur Anbessa Hospital, Ethiopia, Journal of Ethiop.J.Health Dev, 2004, 18(3):199-202
35. Lasserre, R., Treatment of Amebiasis, Journal of Microbiology and Infectious Diseases, 1978, 8(1): 1-4
36. Markell, E., Amebiasis or Disparosis, Journal of Clinical Contributions, 1998, 2: 1-3
37. Mckane, L., & Kandel, J. (1985), Microbiology: Essentials and applications, USA, McGraw-Hill Companies, Inc.
38. Pelczar, M.J., Chan, E.C.S., & Krieg, N.R. (1993), Microbiology concepts and applications, USA, McGraw-Hill Companies, Inc
39. Peritonitis, Retrieved January 05, 2011 from: http://www.healthscout.com/ency/68/473/main.html
40. Repalle,S.S., Yamsani, S.K., Gannu, R & Yamsani, M.R.., Effect of silymarin supplement on the pharmacokinetics of ornidazole in healthy volunteers, Journal of Acta Pharmaceutica Sciencia., 2009, 51: 15-20
41. Symptom of amebiasis, Retrieved January 05, 2011 from: http://www.wrongdiagnosis.com/a/amebiasis/symptoms.htm
42. Salazar, N.P., and Pasay, Ma. C. J., Application of serological procedures in the diagnosis of invasive amebiasis, Journal of Phil J Microbiol Infect Dis, 1991, 29(2): 50-53
43. Salles, J.M., Moraes, L.A., & Salles, M.C., Hepatic amebiasis, Journal of the Brazilian Journal of Infectious Diseases, 2003, 7(2): 96-110
44. Salles, J.M., Salles, M.J., Moraes, L.A., and Silva M.C., Invasive amebiasis: An update on diagnosis and management, Journal of Expert Rev.Anti infect, 2007, 5 (5): 893-901
45. Shargel, L., Mutnick, A. H., Souney, P. F. and Swanson, L. N (2007) Comprehensive Pharmacy Review, 6th Ed, USA, Lippincott Williams & Wilkins

46. Strohl. W.A, Rouse, H., & Fisher, B.D. (2001), Lippincott's illustrated reviews Microbiology, USA, Lippincott Williams and Wilkins
47. Santos, H. L. C., Peralta, R. H. S., Macedo, H. M. D., Barreto, M. G. M & Peralta, J.M., Comparison of Multiplex-PCR and Antigen Detection for Differential Diagnosis of *Entamoeba histolytica,* Journal of Infectious Diseases, 2007, 11(3): 365-370.
48. Shenoy, V.P., Vishwanath, S., Indira, B & Rodrigues, G, Hepato- Pulmonary Amebiasis: a case report, Braz J Infect Dis, 2010, 14 (4): 372-373
49. Smedley, S. R., A method for freeing cultures of *Entamoeba histolytica*, 1956. 50:232-233
50. Von Brand, T., Rees, C. R., Jacobs, L. and Reardon. L. V., Studies on reducing substances and gas formation in cultures of *Entamoeba histolytica* and a single species of symbiotic bacterium, 1943, 37:310-319.
51. Zierdt, C. H., Williams, and R. L *Blastocystis hominis*: axenic cultivation. Exp. Parasitol., 1974, 36:233-243.
52. Chintana ,T., Sucharit ,P., Mahakittikun, V., Siripanth, C., Suphadtanaphongs, W., In vitro studies on the sensitivity of local Entamoeba histolytica to anti-amoebic drugs, Journal of Southeast Asian J Trop Med Public Health.,1986,17(4):591-4.