1. INTRODUCTION

1.1. The disease amoebiasis

*Entamoeba histolytica* is the aetiological agent of amoebiasis, a disease which presents a complex condition ranging from asymptomatic 'carriers' to highly symptomatic and acute infections. It is an ubiquitous disease and is estimated to affect nearly 10 percent of the world's population, although its prevalence and severity may vary from place to place. In general, amoebiasis is more prevalent in tropical regions, where poor sanitation and socioeconomic conditions encourage transmission of the disease. Because of its insidious character, ease of transmission from man to man, its tendency to persist in relatively benign form despite repeated efforts towards clearance of infection, amoebiasis remains one of the major health problems of the world. It has been called the "subtle murderer" by Miller (1941), and yet there are many physicians and public health workers who question whether or not those who harbour asymptomatic strains of *E. histolytica* should be treated. In order to protect not only infected individuals from complications of the disease but others with whom they may come in contact, this question should be answered.

Seven species of anaerobic amoebae are natural parasites of man: *Entamoeba histolytica*, *Entamoeba hartmanni*, *Entamoeba coli*, *Entamoeba gingivalis*, *Endolimax nana*, *Iodamoeba buetschlii* and *Dientamoeba fragilis*. *E. gingivalis* is an inhabitant of mouth, where as the others live in the colon. Of the six species of colon-inhabiting amoebae, only *E. histolytica* is recognised as pathogenic to man. Previously, the terms "small race"
and "large race" were used for *E. histolytica*, but, most of the workers now recognise small race as an independent species of *E. hartmanni*, and the large race as *E. histolytica*. It is the large race which is considered as the aetiological agent of amoebiasis.

1.2. Clinical manifestations of amoebiasis

For convenience of description, the features of amoebiasis may be grouped under two main headings, intestinal and extra-intestinal.

**Intestinal amoebiasis**

Cases of intestinal amoebiasis may be divided into those of acute amoebic dysentery, chronic intestinal amoebiasis and asymptomatic 'carrier' state.

In acute amoebic dysentery, the intestinal manifestations vary from a mild, afebrile diarrhoea to a frank dysenteric attack. The frequency of stools is increased and may vary from 5-10 per day and offensive faecal specimens usually contain blood and mucus. The reaction of the stools is acidic and the microscopic examination of fresh samples reveal the presence of active haematophagous trophozoites of *E. histolytica*. The complications of acute amoebic dysentery include haemorrhage, perforation and peritonitis, appendicitis, pericaecal (pericolic) abscess, gangrene of the large intestine, and invasion of the liver.

Chronic intestinal amoebiasis is, generally, secondary to acute amoebic dysentery, but may also be seen in patients who have never suffered from dysenteric attacks. The chief gastro-intestinal manifestation is an irregularity of the bowel, characterized by the frequent attacks of liquid stools alternating with constipation. Abdomen may reveal thickening of the entire
colon or certain focal areas. In some cases, there may be even a palpable swelling due to the formation of amoebic granulomatous mass or amoeboma.

In the asymptomatic carrier state, there may be no sign of ulceration of the large intestine, but the individual might harbour *E. histolytica* and discharge enormous number of cysts in the formed stools. These carrier cases are responsible for the spread of disease to healthy persons through contamination of food and water supply with cysts.

**Extra-intestinal amoebiasis**

A sizeable proportion of cases suffering from asymptomatic or symptomatic colonic infections develop extra-intestinal amoebiasis, because of the spread of amoebae to other organs, specially liver and the disease is termed as hepatic amoebiasis. Many cases show metastatic spread to other organs such as lungs, pleura, pericardium, brain and peritoneal cavity. The factors which favour the spread of amoebae to extra-intestinal sites, are not well understood. However, delayed diagnosis of these systemic complications often prove fatal.

### 1.3. Chemotherapy of amoebiasis

From the standpoint of chemotherapy, the commonly used amoebicides have been categorized into three groups on the basis of their site of action. The first group includes amoebicides having direct or indirect effect on amoebae in the bowel lumen. The commonly used direct acting drugs are: diiodohydroxyquin, iodochlorhydroxyquin, chiniofon, acetarsone, carbarsone, glycobiarsol, diloxanide furoate, chlorophenoxyamide and paromomycin. The antibiotics tetracycline, chlorotetracycline and oxytetracycline act primarily on the bacterial flora.
and thus exert indirect action on *E. histolytica*. The second group consists of tissue amoebicides that act principally in the bowel wall, liver and other organs. It includes emetine, dehydroemetine and chloroquine. The third group includes metronidazole and other imidazole derivatives which are active at all sites i.e. both in lumen of the large intestine as well as liver and other organs.

1.4. Justification of the present work:

Despite the voluminous work already published on the cultivation of *E. histolytica*, pathogenicity of strains of large race of *E. histolytica* and chemotherapy of amoebiasis, there are still many lacunae in our knowledge of these areas of amoebiasis:

1. Cultivation of *E. histolytica*

(a) According to hypothesis proposed by Diamond (1968a, 1968b), the development of axenic culture from small inocula requires the association of amoebae with trypanosomatids. It is, therefore, generally believed that addition of trypanosomatids is essential for axenization of new strains of *E. histolytica* and for raising axenic cultures from small number of amoebae. Therefore, there is an urgent need to develop methods for initiating axenic cultures from small number of amoebae and to find out whether the hypothesis proposed by Diamond is true or not.

(b) The clone cultures of *E. histolytica* can be useful for characterization of different strains of *E. histolytica* and for determination of degree of heterogeneity in the parental population of any strain. Gillin and Diamond (1978a, 1978b) believe that the clone cultures can be raised by colony formation method in the agar. This
method can not be taken as reliable, because the colonies may arise even from clumps of amoebae in the agar. In view of the importance of clonal cultures for understanding the biological properties of amoebae, a reliable method for developing clonal cultures of axenic *E. histolytica* is a necessary prerequisite.

(c) In the literature, there are contradictory reports on the successful cryopreservation of trophozoites of *E. histolytica*. To obviate the difficulties involved in the maintenance of different strains of *E. histolytica* by frequent subcultures and to avoid possibility of development of culturally induced biological variations in amoebae, there is an urgent need of a simple and reliable method for cryopreservation of amoebae. It would also be valuable for freezing different clones of *E. histolytica*.

(d) Frequent preparation of TP-S-1 medium for maintaining axenic *E. histolytica* as recommended by Diamond (1968b), is quite cumbersome and inconvenient. The stored TP-S-1 medium does not support the growth of *E. histolytica*, because the O-R potential of the medium is unstable and shifts to oxidizing levels after 4-5 days of storage. Therefore, there is an urgent need to devise axenic medium which maintains stable negative O-R potential and which could be stored for a few months without deterioration.

(e) The association of amoebae with trypanosomatids is known to promote the colonization of amoebae and help in the axenization of new strains as well as initiation of small inocula cultures. The effect of trypanosomatids on the growth and metabolism of amoebae in axenic culture needs further investigation.
2. Virulence of *E. histolytica*

(a) In the literature, there are conflicting reports on the virulence of axenic and trypanosomatid associated cultures of *E. histolytica*, which needs evaluation. Further, the role of different bacterial species on the restoration of virulence of axenic cultures as claimed by Wittner and Rosenbaum (1970) also requires confirmation.

(b) Recently some reports have appeared on the restoration of virulence of attenuated and avirulent strains of axenic *E. histolytica* by cholesterol feeding. The possibility of restoring the virulence of axenic (NIH-200 strain) culture of *E. histolytica* by this method should be further evaluated.

(c) Despite the publication of numerous reports on the pathogenicity of different strains of *E. histolytica* isolated from patients and asymptomatic carriers, there is need to develop a simple method for producing experimental amoebiasis in models like hamster, which could be used for studies related to induction of virulence by serial hamster liver passage, and for studies on immunoprophylaxis.

(d) Inspite of extensive studies on the pathogenic activities of *E. histolytica* upon animal hosts, the factors that govern the pathogenesis of the parasite are still largely unknown. The mechanism of enhancement of virulence of *E. histolytica* by cholesterol feeding needs elucidation.

3. Chemotherapy of *E. histolytica*

(a) In most of the laboratories, *E. histolytica* cultures growing with bacterial associates are still employed for *in vitro* screening of drugs. It is well-known that
bacterial cultures of *E. histolytica* do not give reproducible results when used for chemotherapeutic screening. Moreover, the bacterial cultures of *E. histolytica* do not permit us to distinguish between direct versus indirect amoebicidal activities of test compounds. The *in vitro* chemotherapeutic studies on axenic cultures of *E. histolytica* provide a test system for accurate assessment of direct amoebicidal activities of known and new amoebicidal agents. The effect of O-R potential, which largely influences the growth of axenic amoebae, on the amoebicidal activity of drugs needs to be investigated.

(b) Recent studies by Ramachandran *et al.* (1976) have shown that the pus samples from amoebic liver abscess range in pH from 5.2 to 6.7. Stamm (1976) also commented on the acidic nature of diarrhoeal stools. Therefore, it is necessary to discover whether the presently known amoebicidal drugs retain their high amoebicidal activity at acidic pH prevailing in the liver abscess pus and diarrhoeic contents of the bowel or not.

(c) The axenic amoebae in association with trypanosomatids undergo very active phagocytosis in cultures. So far, no studies on the effect of active phagocytosis by amoebae on the amoebicidal activity of drugs have been carried out in axenic culture.

(d) In the clinical practice, a large number of luminal and systemic amoebicides and their combinations are currently being used for treatment of amoebiasis. It is important to discover whether combined regimens used for treating amoebiasis cases possess any direct additive or synergistic action against *E. histolytica* or not.
(e) The methods so far developed for screening amoebicidal agents and detecting the antiamoebic activity of drugs, are largely empirical. Entner and Grollman (1973) attempted to develop a quantitative method for assessing the chemotherapeutic activity of drugs based on the incorporation of labelled amino-acids by the amoebae in the presence of amoebicidal drugs \textit{in vitro}. Anti-amoebic screening based on percentage inhibition of protein synthesis by drugs, thus can provide a very sensitive method for comparing the activities of new and known drugs. Further studies are, therefore, needed to standardize this quantitative method of assessing the antiamoebic activity of drugs.

(f) Since the amoebicidal activities of potential amoebicides discovered by \textit{in vitro} experiments may or may not have direct relevance to their \textit{in vivo} activity, it is necessary to develop experimental models for effective \textit{in vivo} screening of antiamoebic agents. Though several methods of producing experimental hepatic amoebiasis in hamsters are available, there is still a need to develop a simpler method of producing liver infection, by which large number of animals could be infected within a short time. In the methods developed so far, a large proportion of hamsters occasionally die because of bacteraemia caused by large numbers of bacteria inoculated with the amoebic cultures. The method of producing hepatic abscess routinely by inoculation of a very small piece of infected liver lesion has not been used for \textit{in vivo} screening of drugs. Since there are large numbers of trophozoites in the amoebic lesion, it should be possible to infect a large number of animals from a single hepatic abscess of infected hamster. Studies along these lines would be very fruitful and provide an easy method of screening
drugs for activity against hepatic amoebiasis.

In light of the above lacunae in our knowledge of amoebiasis, the following studies with special reference to cultivation, virulence and chemotherapy of *E. histolytica* have been carried out and the results are presented in this thesis:

1. Cultivation
   (a) Initiation of axenic cultures from small inocula without trypanosomatids associates.
   (b) Development of clonal cultures of *E. histolytica*.
   (c) Successful cryopreservation of *E. histolytica* strains.
   (d) Development of axenic medium which can support growth of amoebae even after long term storage.
   (e) Effect of trypanosomatids on the growth of axenic *E. histolytica*.

2. Virulence
   (a) Virulence of axenic and monoxenic cultures of *E. histolytica* growing with trypanosomatids.
   (b) Role of bacterial flora in restoration of virulence of axenic *E. histolytica*.
   (c) Influence of cholesterol on the virulence of axenic amoebae.
   (d) Production of hepatic amoebiasis in hamster by serial infected liver passage.
   (e) Mechanism of pathogenicity of *E. histolytica*.

3. Chemotherapy
   (a) Influence of pH on the amoebicidal activity of drugs.
   (b) Effect of active phagocytosis on the amoebicidal action of drugs against axenic *E. histolytica*.
(c) Effect of redox-potential and amoebic inocula on amoebicidal action of drugs.

(d) Combined action of drugs on *E. histolytica*.

(e) Standardization of a new method for *in vitro* screening of drugs, by inhibition of protein synthesis.

(f) Development of an improved experimental method for *in vivo* screening of amoebicidal drugs against hepatic amoebiasis.