COMPARISON OF *ENTAMOEBA HISTOLYTICA* ANTIBODIES AND LECTIN ANTIGEN LEVELS IN THE SERA OF PATIENTS SUSPECTED WITH AMOEBIC LIVER ABSCESS IN HUSM

By

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ABSTRAK

Pengenalan: Ujian hemaglutinasi tak langsung (IHA) adalah salah satu ujian serologi yang digunakan secara meluas untuk mengesan antibodi terhadap *Entamoeba histolytica* di dalam darah pesakit. Di kawasan endemik penyakit amebiasis seperti di negeri Kelantan, keputusan IHA yang positif adalah sukar untuk ditafsirkan. Ujian ELISA TechLab *E. histolytica* II adalah kit komersial untuk mengesan kehadiran antigen spesifik terhadap *Entamoeba histolytica* di dalam najis pesakit. Kit ini juga telah dilaporkan berupaya mengesan antigen tersebut di dalam sampel darah pesakit abses hati amebik. Objektif kajian ini adalah untuk mengetahui dan membandingkan ujian pengesanan antibodi (IHA) dan ujian pengesanan antigen (ELISA) di dalam serum pesakit abses hati amebik.

Metodologi: Kajian ini telah dilakukan secara hirisan lintang ('cross sectional'). Seramai 43 pesakit abses hati amebik yang dimasukkan ke wad surgeri, HUSM telah terlibat dalam kajian ini. Diagnosa pesakit dibuat berdasarkan tanda-tanda klinikal dan keputusan ultrasound atau imbasan CT ('Computed Tomography'). Data klinikal pesakit di ambil daripada fail pesakit. Sampel darah mereka telah diambil untuk dilakukan kedua-dua ujian IHA dan ELISA mengikut arahan prosedur yang telah ditetapkan.

Keputusan kajian: Kami mendapati bahawa majoriti pesakit adalah di kalangan bangsa Melayu, kaum lelaki lebih ramai mengidap penyakit abses hati berbanding kaum wanita dan kebanyakan berumur di antara 20 hingga 59

tahun. Tiga tanda-tanda penyakit abses hati oleh pesakit adalah demam, sakit di bahagian abdomen dan bengkak hati. Kebanyakan pesakit mempunyai satu ketulan abses sahaja pada bahagian kanan hati atau hepar serta meningkat bilangan sel darah putih. Daripada 43 orang, 76.7% pesakit adalah positif untuk ujian IHA dan hanya 2.3% pesakit positif untuk ujian ELISA. Perbandingan antara kedua-dua ujian tersebut telah menunjukkan persetujuan keputusan yang lemah. Keputusan ujian ELISA juga tidak mempunyai hubungan atau kaitan dengan keputusan ujian IHA untuk setiap paras antibodi. Daripada kajian ini juga didapati bahawa komplikasi penyakit ini yang di hidapi oleh 30.2% pesakit adalah selaput paru-paru berair ('pleural effusion').

Rumusan: Berdasarkan keputusan yang diperolehi, ujian ELISA adalah tidak sensitif untuk mengesan antigen *E. histolytica* di dalam sampel serum daripada pesakit abses hati amebik. Tambahan pula, didapati bahawa keputusan ujian ELISA tidak selari dengan keputusan paras antibodi daripada IHA. Oleh yang demikian, ujian ELISA (TechLab *E. histolytica* II) tidak sesuai digunakan untuk ujian serologi bagi pesakit abses hati amebik di HUSM.

Comparison of *Entamoeba histolytica* antibodies and lectin antigen levels in the sera of patients suspected with amoebic liver abscess (ALA) in HUSM

ABSTRACT

Background: Indirect haemagglutination assay (IHA) is one of the most widely used methods to detect *Entamoeba histolytica* antibodies. In an amoebiasis endemic area such as Kelantan, interpretation of a positive IHA result can be problematic due to the high background antibody levels. The TechLab *E. histolytica* II ELISA is a commercial kit for detection of specific Gal/GalNAc lectin antigen in stool samples, and has been reported to be able to detect the antigen in serum samples from patients with amoebic liver abscess (ALA). Thus in this study, the usefulness of the TechLab *E. histolytica* II ELISA was compared with IHA in the diagnosis of ALA. The objective of the study is to determine and compare the level of antibodies by IHA and the presence of Gal/GalNAc lectin antigens by ELISA in the sera of suspected ALA patients.

Method: This is a cross sectional study involving 43 clinical or suspected ALA patients who were admitted to the surgical ward, Hospital USM, Kelantan. The diagnosis of ALA was established based on clinical symptoms and signs, ultrasound and/or CT scan results. Clinical data of the patients were reviewed from the hospital files. The serum specimens were obtained from the patients and tested with IHA and ELISA methods according to the manufacturer's instructions.

Results: The majority of patients in this study were Malays, males were affected more than females and the majority was in the age group of 20-59 years old. Three most common clinical signs and symptoms on presentation were fever, abdominal pain and hepatomegaly. Majority of patients were found to have single abscess located at the right lobe of liver and with the presence of leucocytosis. Of all 43 patients, 76.7% was IHA positive and only 2.3% was ELISA positive. The agreement between IHA for antibody detection and ELISA for lectin antigen detection was poor. There was no correlation between ELISA results and IHA of different antibody titers. The complication of ALA was pleural effusion occurring in 30.2% of the patients.

Conclusion: Based on the findings, the ELISA method used in this study was not sensitive in detecting amoebic antigen in serum samples from ALA patients. In addition, the results of ELISA test did not correlate with the IHA antibody titers. Therefore, the TechLab *E. histolytica* II ELISA was found not to be a useful test for serological diagnosis of ALA in HUSM.

CHAPTER ONE

INTRODUCTION

1.1 Statement of the problem

Amoebiasis is the most aggressive protozoal disease caused by the protozoan parasite *Entamoeba histolytica* that affects the human bowel, considered the second or third leading cause of death amongst the parasitic diseases, surpassed only by malaria and schistosomiasis. It is a common worldwide disease; 100,000 people are estimated to die each year from amoebic colitis and amoebic liver abscess (ALA). Estimated worldwide prevalence of ALA is about 50 million infections per year (WHO, 1997).

The diagnosis of ALA is sometimes difficult since its clinical manifestations are highly variable. In areas of endemicity, ALA should always be suspected in a patient with fever, weight loss, and upper quadrant abdominal pain and tenderness. Imaging techniques such as ultrasonography, computerized tomography, and magnetic resonance imaging have excellent sensitivity for the detection of liver abscess arising from any cause but cannot distinguish amoebic abscesses from pyogenic (bacterial) abscesses or necrotic tumors. Once suspected clinically, ALA requires specific treatment without delay, thus this necessitates a reliable diagnostic test.

The definitive confirmation of hepatic amoebiasis is based on a demonstration of *Entamoeba histolytica* trophozoites in the aspirated pus, or more frequently from the necrotic material obtained by needle biopsy of the edge or the bottom of the lesion. Nevertheless, the amoeba will be found in only a small percentage of cases. Most patients with an ALA do not have coexistent amoebic colitis. Therefore, stool microscopy or antigen detection in stool samples is not helpful for diagnosis; less than ten percent of patients have identifiable amoebae in stool (Haque *et al.*, 2000).

Serology has become a valuable tool for diagnosis, detecting specific circulating antibodies against the invasive forms. Among the variety of serological tests available nowadays, indirect hemagglutination (IHA), even when used in serological studies, is a very sensitive assay, being positive in 90 to 100% of patients with liver amoebiasis. The drawbacks of antibody detection are that the antibody may not be detectable in the early phase of the infection and is known to persist for many months following a resolution of acute ALA. Thus the presence of an antibody may or may not signify an acute infection (Salles *et al.*, 2003).

The amoebic serology which is currently performed at the Department of Medical Microbiology and Parasitology, HUSM is based on antibody detection by IHA. It is a good and useful test but often may be difficult to interpret in endemic areas when there is a high background level of seropositivity for amoebiasis. It is not clearly known what level of endemicity exist in Kelantan,

thus interpretation of amoebic serology can be uncertain especially if the antibody titer is not high. In 2003, the number of requests for amoebic serology was 108 and 68 (62.9%) cases were detected as positive by IHA (Cellognost; Behring Diagnostics, Marburg, Germany) where the antibody titers were equal or more than 1:256.

On the other hand, the antigen detection test has been proven to be a useful tool to confirm acute infection either in a very early phase of the infection or when the antibody has been produced. It may provide great advantages in endemic areas where there is a high prevalence of serum anti-amoebic antibodies.

Several research groups have reported the detection of amoebic antigen in the serum of liver abscess patients. For example, Abd-Alla and colleagues (1993) detected the Gal/GalNAc lectin in the sera of 75% of South African patients with ALA. A commercially available antigen detection test, the TechLab, Inc. (Blacksburg, Va.), *E. histolytica* test, detects the Gal/GalNAc lectin in stool samples and has been proven to be a sensitive and specific means of diagnosis of colitis (Haque *et al.*, 1995). A second-generation kit that uses an improved capture antibody has recently been developed by TechLab. Haque *et al.* (2000) has evaluated this improved second generation kit for detection of lectin antigen in the serum of ALA patients in Dhaka, Bangladesh and it was found that 96% of them were positive for Gal/GalNAc lectin when tested prior to treatment with metronidazole.

Based on the previous study (Haque *et al.*, 2000), we compare the kit (TechLab *E. histolytica* II test) with the current IHA test that is being used in HUSM for serological diagnosis of ALA patients. This antigen-based test may play an important role in serological diagnosis of ALA in the near future as supported by data from the research above. It may provide a more reliable result than the antibody-based detection that is currently being used in our setting.

1.2 Aim of study

1.2.1 General objective

To determine and compare the level of *Entamoeba histolytica* antibodies and the presence of Gal/GalNAc lectin antigens in the sera of suspected ALA patients.

1.2.2 Specific objectives

1.2.2.1 To determine the level of *Entamoeba histolytica* antibodies in the sera of suspected ALA patients by IHA method.

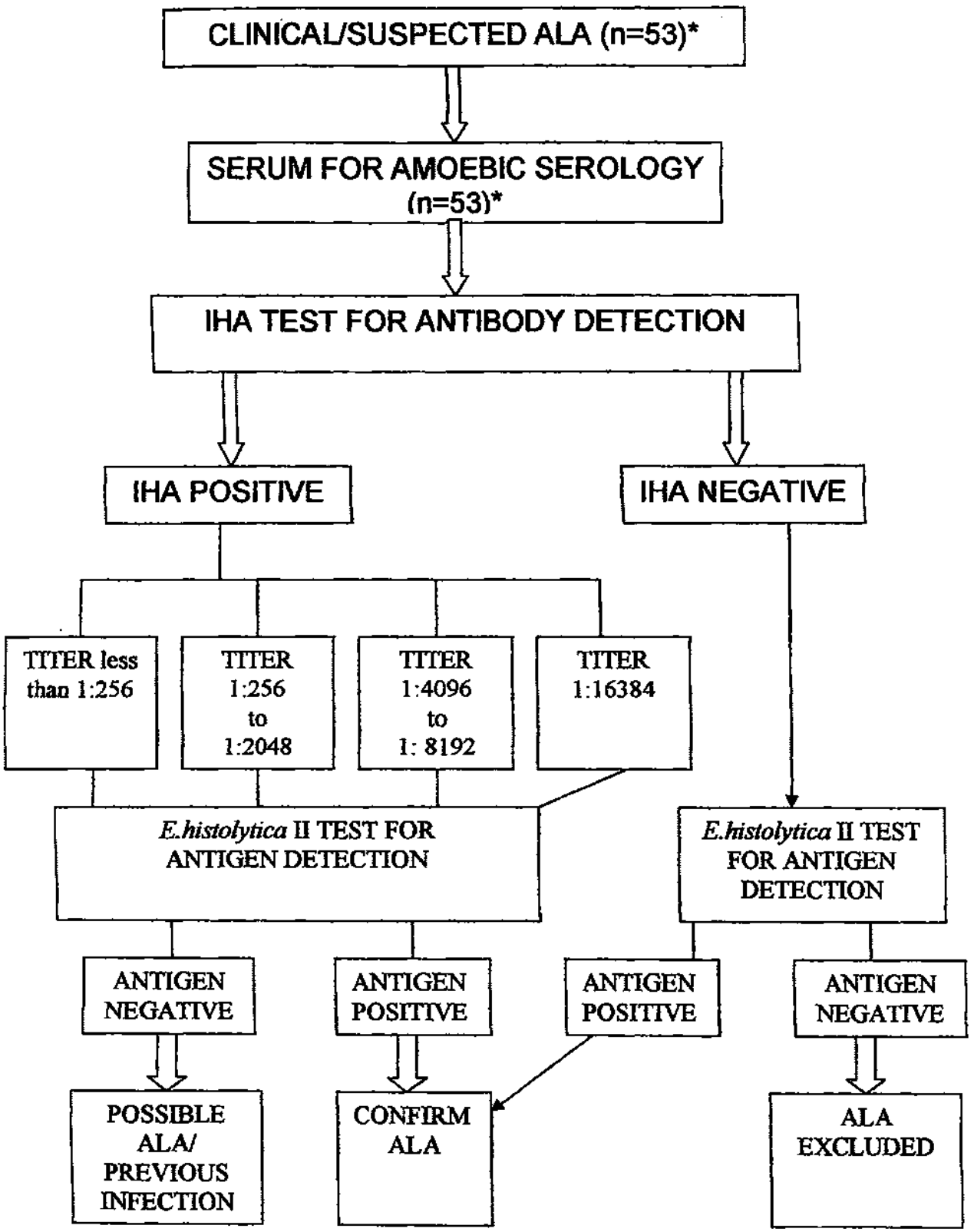
1.2.2.2. To determine the presence of Gal/GalNAc lectin antigens in the sera of suspected ALA patients by ELISA (TechLab) method.

1.2.2.3 To compare the presence of Gal/GalNAc lectin antigens (by ELISA) and the level of antibodies (by IHA) in the sera of suspected ALA patients.

1.2.2.4 To compare the presence of Gal/GalNAc lectin antigens in different antibody titers of suspected ALA patients.

The study has been approved by the Scientific Committee of Research and Ethics, School of Medical Science, USM on 30th August 2004. A short-term IRPA grant (A/C: 304/PPSP/6131418) was obtained in realizing this project.

1.3 Work flow chart



*calculated sample size

Figure 1.1. Work flow chart of the study.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Entamoeba histolytica*: An overview of the organism's biology

All species in the genus of *Entamoebae* are recognized as colonizing the human large intestine but *Entamoeba histolytica* is the only species known to cause disease in humans. For this reason it is by far the most widely studied intestinal protistan parasite, with a literature spanning over 120 years (Clark *et al.*, 2000).

It belongs to the subphylum Sarcodina, class Lobosea, and family Entamoebidae. Humans appear to be the primary host, but several non-human primates, cats, dogs and rats have also been documented as occasional hosts. With few exceptions, all *Entamoeba* species have a simple life cycle consisting of an infective cyst stage and a multiplying trophozoite stage. Within the genus *Entamoeba*, species variously produce cysts with one, four or eight nuclei (figure 2.1) (Tanyuksel and Petri, 2003).

In *E. histolytica*, the cyst is ingested via faecally contaminated food or water and passes through the stomach and small intestine. The trophozoite emerges in the terminal ileum or as the cyst enters the large intestine where it establishes the new infection. Trophozoites ingest bacteria and multiply by binary fission in the colon where, in response to unknown stimuli, they re-

encyst. Cysts are shed periodically in the stool thus completing the cycle (Clark *et al.*, 2000).

Trophozoites are short-lived outside the body and do not survive passage through the upper gastrointestinal tract. In contrast, cysts are resistant to chlorination, gastric acidity and dessication, and may remain viable outside the host for weeks or months in a humid environment and stay infective for several days. Cysts are rapidly destroyed at temperatures under -5°C and over 40°C (Tanyuksel and Petri, 2003).

It is the mature cyst that, when consumed in contaminated food or water, is infectious. The invasive form of the parasite is the trophozoite which can penetrate the intestinal mucosa and disseminate to other organs. Cysts do not develop within tissues (Tanyuksel and Petri, 2003).

The size of the trophozoites ranges from 10 µm to 60 µm in diameter. Locomotion is by means of a single well defined pseudopodium. There is normally a single nucleus. The cytoplasm often has ingested red blood cells when the trophozoites are isolated from symptomatic individuals. Sometimes leukocytes and bacteria are visible. The cytoplasm is rich in glycogen and has ribosomes arranged in helices which aggregate. There are no classical mitochondria, rough endoplasmic reticulum, or Golgi apparatus visible. The nucleus is spherical, measuring 4 µm to 7 µm in diameter, consisting of a delicate achromatic membrane lined usually by a single layer of small chromatin granules, uniform in size, in contact or very close to each other. The

nucleus has a small spherical karyosome (0.5 µm in diameter), is often centrally located, and surrounded by an achromatic capsule-like structure (Clark *et al.*, 2000).

E. histolytica cysts are round or slightly oval hyaline bodies measuring 10 µm to 16 µm in diameter and surrounded by refractile walls. The mature cyst contains four nuclei. Cyst nuclei are morphologically similar to those of trophozoites, but are smaller in the mature cyst. When stained with iron-hematoxylin, the cyst cytoplasm appears vacuolated with numerous glycogen deposits that decrease in size and number as the cyst matures. Chromatoid bodies, which are aggregated ribosomes, can be identified inside the cytoplasm as rod shaped structures with blunt or rounded ends. These disappear as the cyst matures (Clark *et al.*, 2000).

Like other protozoa, *E. histolytica* appears incapable of de novo purine synthesis. Biochemical analysis has indicated that glutathione is not present. For this reason, *E. histolytica* is different from higher eukaryotes. It also uses pyrophosphate instead of ATP (Tanyuksel and Petri, 2003).

The gene organization of *E. histolytica* seems quite distinct from that of other eukaryotes. The ribosomal RNA (rRNA) genes are located on extrachromosomal circular DNA molecules (Bhattacharya *et al.*, 1998). Although the structure of *E. histolytica* chromosomes is not yet known completely, electrokaryotypic analysis suggests that depending on the isolate used, 31-35 chromosomes are identified ranging in size from 0.3 to 2.2

megabases and they give a total haploid genome size of approximately 20 megabases (Willhoeft and Tannich, 1999). A complete sequence map of the ribosomal DNA (rDNA) episome has been successfully completed. Sehgal *et al.* (1994) found that *E. histolytica* circular DNA is 24.5 kilobases. This sequence has proved quite useful for genotyping of the different enteric amoebae.

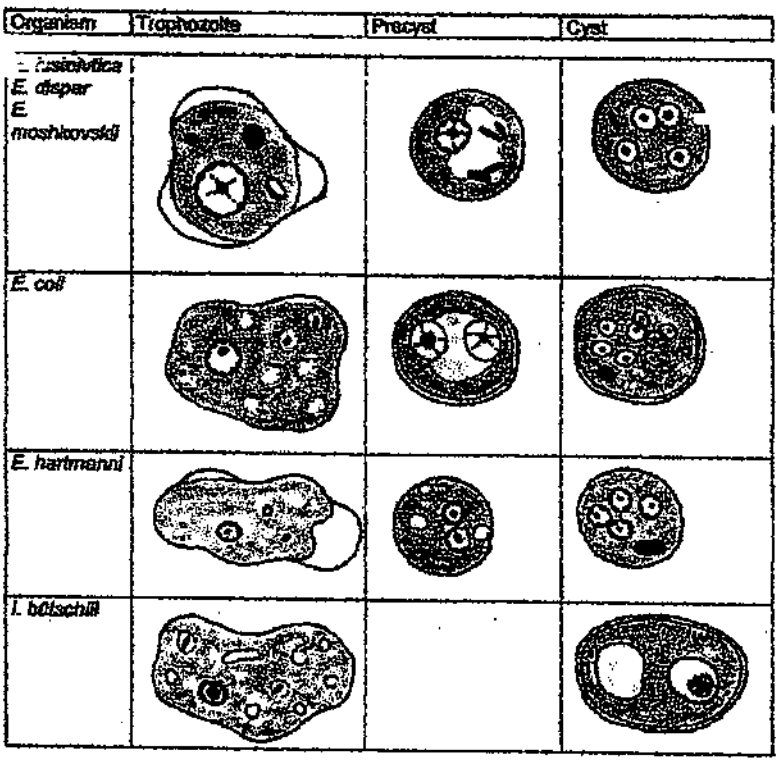


Figure 2.1. Drawing of intestinal *Entamoeba spp.* showing morphological features

2.2 Epidemiology and transmission

In the light of earlier reports about the prevalence of amoebiasis, interpretation is very difficult because older data did not differentiate between morphologically

identical species, one that is noninvasive (*E. dispar*) and one that is invasive (*E. histolytica*), but they have a high degree of divergence (Tanyuksel and Petri, 2003).

In 1986, Walsh assessed the existing global prevalence data and concluded that in 1981, 480 million people were infected with *E. histolytica* worldwide. Approximately 36 million developed disabling colitis or extraintestinal abscesses with at least 40,000 deaths each year, resulting in amoebiasis ranking third, after malaria and schistosomiasis, as a parasitic cause of death. However the epidemiological data quoted did not distinguish between *E. dispar* and *E. histolytica*. In 1997, the World Health Organization reported that *E. histolytica* is responsible for up to 100,000 deaths annually. The vast majority of these infections are acquired in the developing world.

E. histolytica and *E. dispar* have a worldwide distribution, being found in cold, temperate, and tropical climates. Both are more prevalent in disadvantaged communities and in tropical areas. This is believed to be related to poorer sanitation, low socioeconomic status, crowding and decreased resistance in those living in these environments. Recognized high-risk areas for acquiring amebiasis, include Mexico, the western portion of South America, West Africa, South Africa (particularly in the black population), parts of the Middle East and South and Southeast Asia. Invasive disease seems to be more common in these areas (Jackson, 2000).

In Mexico, a national serosurvey demonstrated that 8.4% of the population was exposed to invasive amoebiasis, representing one million cases of the disease (Salles *et al.*, 2003). In some tropical countries, antibody prevalence rates (reflecting past or recent infection) exceed 50% (Ravdin and Stauffer, 2005). In Malaysia, amoebiasis is an endemic disease. The prevalence, as demonstrated by stool examination for cysts which had been carried out by several workers on various populations in all ethnic and age group ranged from 1.0% to 40.7% (Jamaiah and Shekhar, 1999).

In industrialized countries, amoebiasis occurs in sexually active homosexual men, immigrants, tourists who travel to areas of endemic infection, institutionalized persons, and human immunodeficiency virus HIV-positive individuals (Tanyuksel and Petri, 2003). The overall prevalence of *E. histolytica* infection in industrialized countries such as the United States has been estimated to be 4%, with certain high-risk groups having a much higher incidence of infection and disease (Ravdin and Stauffer, 2005). Many of the cases identified in North America and in Europe are imported, but a level of endemicity is present and occasional water-borne epidemics have occurred (Jackson, 2000).

In a study of asymptomatic carriers of *E. histolytica*, infections with this organism were equally common in males and females. Asymptomatic carriers are more likely to spread the disease than symptomatic patients with invasive amoebiasis. The necessity to identify and treat asymptomatic carriers is

emphasized by the observation that 10% of them develop invasive amoebiasis (Gathiram and Jackson, 1987).

Expression of disease varies with geographic location. For example, in Egypt the predominant presentation is amoebic colitis, whereas in South Africa there is an excessive rate of amoebic liver abscess (ALA) (Ravdin and Stauffer, 2005). In an endemic area in Vietnam, the incidence of ALA was noted to be at least 21 per 100,000 inhabitants per year (Blessmann *et al.*, 2002a). In Malaysia, a number of hospital based studies have been carried out on ALA. For example, ALA was found in 44.1% of liver abscess patients (Goh *et al.*, 1987) and 39% of patients with amoebiasis (Jamaiah and Shekhar, 1999).

ALA was found to be rare in children, since more than 95% of all ALA patients were adults (more than 15 years old) with a peak incidence in the 30–49-year-old age groups. In addition, incidence of ALA was sex dependent and was much higher in males. The male to female ratio in adult patients was approximately 7:1 for the 30–49-year-old age groups and approximately 2.5:1 for the age groups more than 60 years old (Blessmann *et al.*, 2002a). Until now, it remains unclear why hepatic amoebiasis is more common in men than in women. Despite the higher incidence of ALA in males, the parasitologic and seroepidemiologic survey revealed a significant higher infection rate for intestinal protozoon parasites, including *E. histolytica* in females (Blessmann *et al.*, 2002a).