COMPARISON OF CRUDE SOLUBLE ANTIGEN (CSA)-ELISA AND EXCRETORY SECRETORY ANTIGEN (ESA)-ELISA FOR SERODIAGNOSIS OF INVASIVE AMOEBIASIS IN SELECTED ORANG ASLI SERUM SAMPLES

by

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LIST OF SYMBOLS AND ABBREVIATIONS

~	About
%	Percentage
<	Less than
≥	Greater than or equal to
°C	Degree Celsius
µg	Microgram
µl	Microliter
Ab	Antibody
Ag	Antigen
ALA	Amoebic liver abscess
CSA	Crude Soluble Antigen
cm	Centimeter
dH_2O	Distilled water
ESA	Excretory secretory antigen
ELISA	Enzyme-linked immunosorbent assay
et al.	*et alii* – 'and others'
X *g*	Gravity force
h	Hour
HCL	Hydrochloric acid
IHA	Indirect hemagglutination assay
kDa	Kilodalton
L	Litre

mA	Miliampere
min	Minute
ml	Mililitre
nm	Nanometer
OD	Optical density
PBS	Phosphate buffered saline
PBST	PBS-Tween 20
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SD	Standard deviation
sec	Seconds
TMB	3,3',5,5'-tetramethylbenzedine

PERBANDINGAN ANTIGEN LARUT MENTAH (CSA)-ELISA DAN ANTIGEN KUMUHAN-REMBESAN (ESA)-ELISA UNTUK SERODIAGNOSIS JANGKITAN AMEBA INVASIF MENERUSI SAMPEL SERUM ORANG ASLI TERPILIH

ABSTRAK

Amebiasis ialah jangkitan yang disebabkan oleh *Entamoeba histolytica*, sejenis parasit protozoa yang membunuh kira-kira 100,000 manusia setiap tahun dan jangkitan ini merupakan punca kematian kedua tertinggi akibat parasit protozoa selepas malaria. Amebiasis invasif wujud dalam dua bentuk iaitu sama ada amebiasis di dalam usus ataupun amebiasis di luar usus yang mana manifestasi yang paling biasa ialah abses hati ameba (ALA). Di Hospital Universiti Sains Malaysia (Hospital USM), diagnosis ALA bergantung kepada kombinasi penemuan klinikal, pemeriksaan ultrasound hati dan serodiagnosis menggunakan kit komersial melalui teknik asai hemagglutinasi tidak langsung (IHA). Asai imunojerapan berpaut enzim (ELISA) ialah asai kuantitatif rutin makmal yang mudah dilaksanakan, sensitif dan boleh dipercayai. CSA merupakan antigen yang digunakan secara meluas di dalam ELISA untuk serodiagnosis ALA dan juga di dalam kit ujian komersial IHA, manakala ESA dilaporkan sangat sensitif dalam kajian-kajian kebelakangan ini. Dalam kajian awal ini, keberkesanan CSA-ELISA dan ESA-ELISA dibanding berdasarkan ujian standard IHA yang komersil. Sebelum membanding kedua ELISA tersebut, sampel serum Orang Asli disaring menerusi kit IHA dengan nilai potong pada titer 1:256 dan 33 sampel positif dan 30 sampel negatif telah diperolehi dari bank sampel serum

kami. Berdasarkan nilai potong min densiti optik (OD) + 2SD, CSA-ELISA menunjukkan 90.9% persetujuan positif (sensitiviti), manakala ESA-ELISA hanya menunjukkan 60.6% persetujuan positif. Walau bagaimanapun, CSA-ELISA dan ESA-ELISA kedua-duanya menunjukkan lebih 96% persetujuan negatif (spesifisiti).

COMPARISON OF CRUDE SOLUBLE ANTIGEN (CSA)-ELISA AND EXCRETORY SECRETORY ANTIGEN (ESA)-ELISA FOR SERODIAGNOSIS OF INVASIVE AMOEBIASIS IN SELECTED ORANG ASLI SERUM SAMPLES

ABSTRACT

Amoebiasis is an infection caused by protozoan parasite *Entamoeba histolytica* which is responsible for about 100,000 deaths per year and it is the second leading cause of death due to protozoan parasites after malaria. Invasive amoebiasis can be categorized into either intestinal amoebiasis or extraintestinal amoebiasis, and the most common manifestation of invasive amoebiasis is amoebic liver abscess (ALA). At Hospital Universiti Sains Malaysia (Hospital USM), diagnosis of ALA relies on a combination of clinical findings, ultrasound examination of the liver and serodiagnosis using commercial kit such as indirect hemagglutination assay (IHA) technique. ELISA is a quantitative, routine laboratory assay which is simple to perform, sensitive and reliable. CSA is widely used in ELISA for serodiagnosis of ALA and also in commercial IHA test kit, while ESA was reported to be highly sensitive in recent studies. In this preliminary study, we compared CSA-ELISA and ESA-ELISA in relation to the standard commercial IHA test. Prior to performing ELISA, we screened selected Orang Asli serum samples by IHA with cutoff at 1:256 and obtained 33 positive samples and 30 negative samples. Based on the cutoff value of mean optical density + 2SD, CSA-ELISA showed 90.9% positive agreement (sensitivity), while ESA-

ELISA only showed 60.6% positive agreement. In contrast, both CSA-ELISA and ESA-ELISA showed over 96% negative agreement (specificity).

CHAPTER 1

1. Introduction

1.1.Background of study

Entamoeba histolytica is a protozoan parasite. There are two forms of *E. histolytica*, either the infectious cyst form or the amoeboid trophozoite stage (Stanley, 2003). *E. histolytica* is the causative agent for amoebiasis, including invasive intestinal amoebiasis and extra-intestinal amoebiasis. Molecular epidemiology of amoebiasis showed 3.2% (16/500) of Malaysian aborigines were infected with *E. histolytica* (Tengku *et al.*, 2012). There are several modes of transmissions such as ingestion of contaminated food and water with cyst of *E. histolytica*, travelling to endemic areas, as well as practicing oral and anal sex (Tengku & Norhayati, 2011; Stanley, 2003).

Invasive amoebiasis can be either intestinal or extraintestinal. The most common manifestation of intestinal amoebiasis is the amoebic colitis. Amoebic colitis patients give symptoms such as bloody diarrhea as well as abdominal pain and tenderness. Diagnosis of amoebic colitis is often based on commercially available Double Sandwich Enzyme-linked Immunosorbent Assay (ELISA) that detects *E. histolytica* antigens in stool as the microscopy technique cannot distinguish *E. histolytica* and *E. dispar* (Stanley, 2003). Amoebic Liver Abscess (ALA) is an inflammatory space-occupying lesion that usually occurs in the right lobe of the liver and it is the common manifestation of extra-intestinal amoebiasis. People within the age group of 20 to 45 years and male (nine times more

common from female) are the most common group of people with ALA. Their common clinical presentations include abdominal pain, fever and anorexia. In addition, cough with or without sputum and pleuritic chest pain are also seen in ALA patients (Sharma & Ahuja, 2003).

There are a few ways of diagnosing ALA such as by ultrasound, needle aspiration, detection of antigenic proteins through polymerase chain reaction (PCR) and detection of antibodies through enzyme-linked immunosorbent assay (ELISA) (Sharma & Ahuja, 2003). ELISA is a suitable routine diagnostic test because of its simplicity, sensitivity and reliability (Tan *et al.*, 2013). Currently, the diagnosis for ALA is still based on the results of clinical manifestations, radiology imaging, serology and when these three methods indicate ALA, liver abscess aspiration should be performed for confirmation (Wong *et al.*, 2011). Crude soluble antigens (CSA), excretory secretory antigens (ESA), plasma membrane antigens, purified antigenic proteins or recombinant proteins can be used as the amoebic antigens for detection of antibody in serological assays such as ELISA (Tan *et al.*, 2013).

1.2. Objectives of study

1.2.1. General objective

The general objective of the study is to compare the efficacy of crude soluble antigen (CSA)-ELISA and excretory secretory antigen (ESA)-ELISA for serodiagnosis of invasive amoebiasis in selected Orang Asli serum samples.

1.2.2. Specific objectives

1. To compare the positive percentage agreement of CSA-ELISA and ESA-ELISA for serodiagnosis of invasive amoebiasis in selected Orang Asli serum samples.
2. To compare the negative percentage agreement of CSA-ELISA and ESA-ELISA for serodiagnosis of invasive amoebiasis in selected Orang Asli serum samples.

CHAPTER 2

2. Literature review

2.1. Entamoeba histolytica

Genus of *Entamoeba* consists of six species that are found in human intestine; *Entamoeba histolytica*, *E. dispar*, *E. moshkovskii*, *E. coli*, *E. hartmanni* and *E. polecki*. Only *E. histolytica* causes human disease whereas the others are the commensals parasites (Ngui *et al.*, 2012). *E. histolytica* is a protozoan parasite that has a simple life cycle. It exists in two forms; the cyst form and the trophozoite stage. The cysts are round in shape, having a diameter of 10 – 15 μm and they are surrounded by refractile wall. Besides that, they contain four nuclei, glycogen and chromatoid bodies (Figures 2.1 & 2.2) (Stanley, 2003). The nucleus has a central karyosome and peripheral granules.

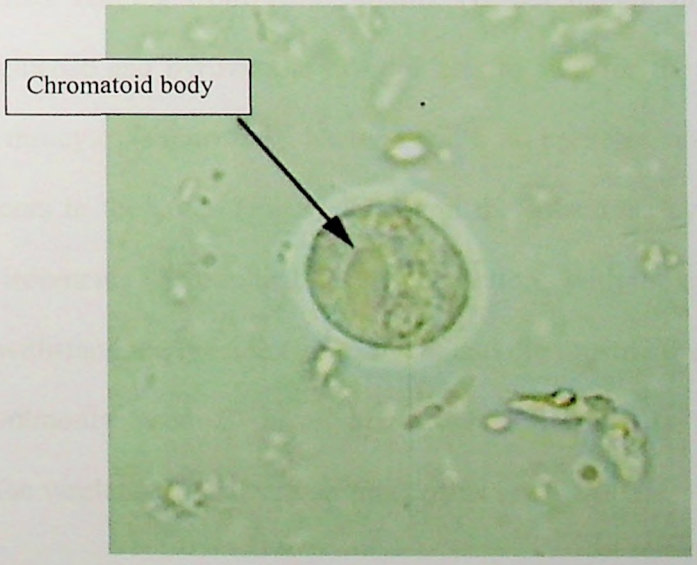

Figure 2.1: Unstained cyst of *E. histolytica/E. dispar*. The chromatoid body has a blunt and rounded ends.

Source: www.cdc.gov/dpdx/amebiasis/index.html

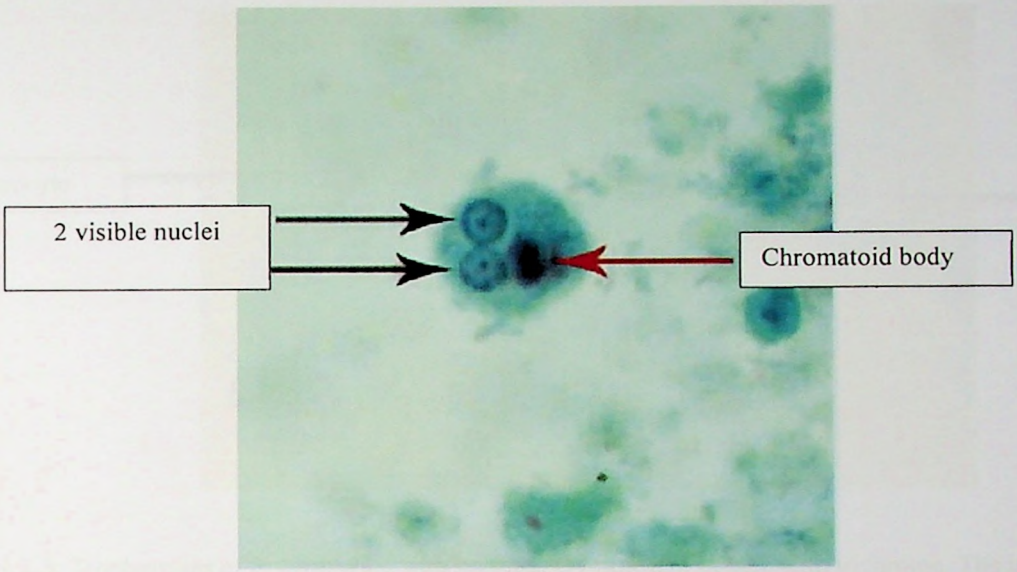

Figure 2.2: Cyst of *E. histolytica/E. dispar* stained with trichrome. Two nuclei and a chromatoid body with typically blunted ends. Source: www.cdc.gov/dpdx/amebiasis/index.html

E. histolytica trophozoites are highly motile. They are pleomorphic in shape, having a varying diameter ranging from 10 – 50 μm. By not having mitochondria, anaerobic conversion of glucose and pyruvate to ethanol acts as fuel for the highly motile motion. They ingest erythrocytes (Figure 2.3), bacteria and food particles, and reproduce by binary. Encystment occurs in the colon (Figure 2.4), and the infectious cysts are excreted in the stool to the environment. The trophozoites may exit along with the cysts in the stool but the former cannot withstand the outside environment and die outside the human body (Stanley, 2003). The commonly used *E. histolytica* trophozoite that is axenically cultured in laboratories is the virulent HM1:IMSS strain (Loftus *et al.*, 2005).

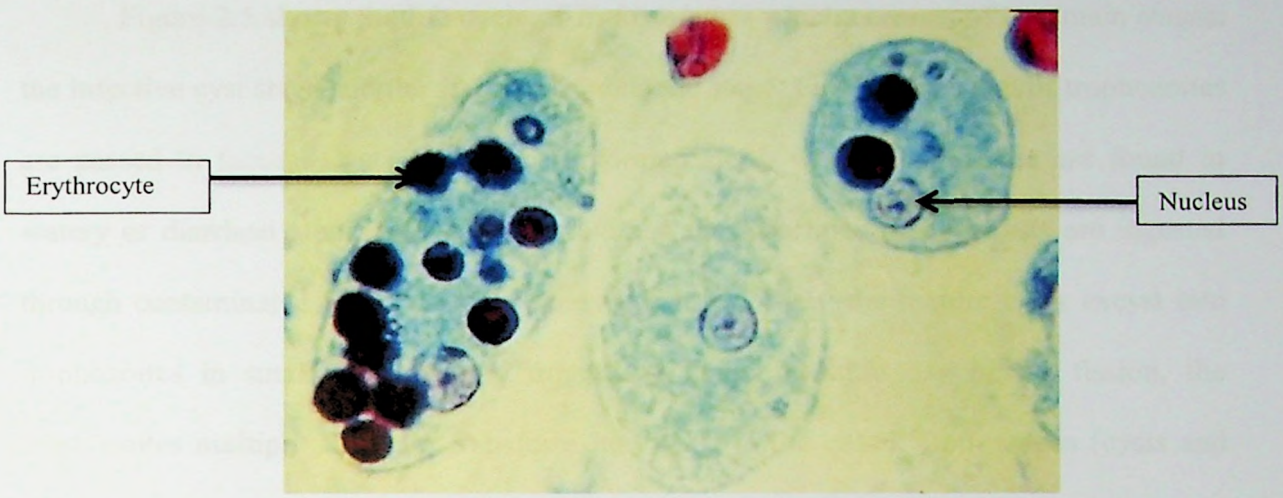

Figure 2.3: Trophozoites of *E. histolytica* with ingested erythrocytes stained with trichrome. The ingested erythrocytes appear as dark inclusions. The parasites above show nuclei that have the typical small, centrally located karyosome, and thin, uniform peripheral chromatin. Source: www.cdc.gov/dpdx/amebiasis/index.html

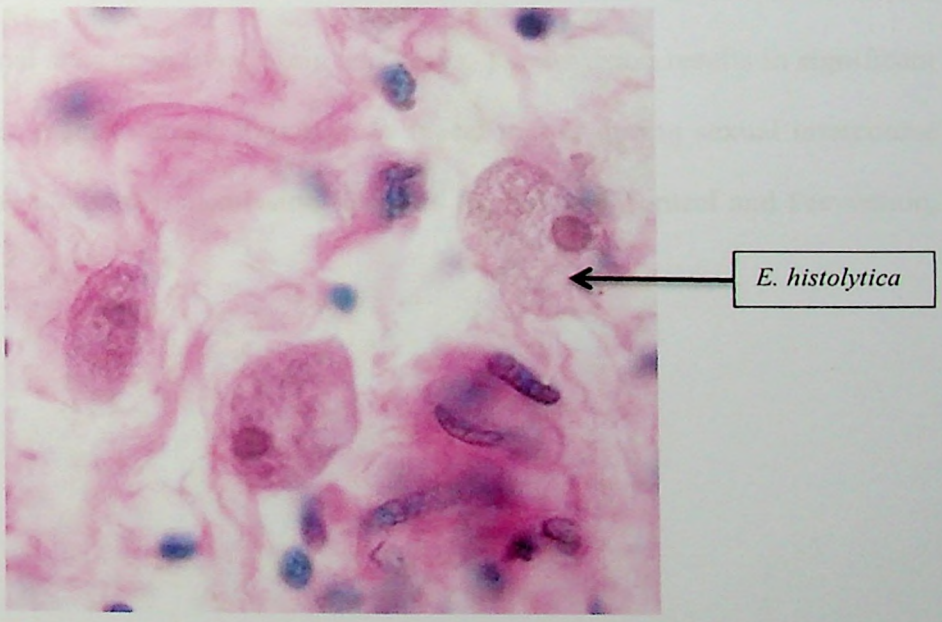

Figure 2.4: *Entamoeba histolytica* trophozoites in colon tissue stained with H&E. Source: www.cdc.gov/dpdx/amebiasis/index.html

Figure 2.5 shows the life cycle of *E. histolytica* which consists of two main stages: the infective cyst stage and the dividing trophozoite stage. Firstly, cysts and/or trophozoites are passed in faeces. Cysts are found in formed stool, while trophozoites are found in watery or diarrheal stool. Infection occurs when the infectious mature cysts are ingested through contaminated food, water or hand with feces. Then, the mature cysts excyst into trophozoites in small intestine and migrate to large intestine. By binary fission, the trophozoites multiply and later transform into cysts at the colon. Both stages (cysts and trophozoites) are passed in the faeces. The cysts can survive up to weeks in the external environment because of their thick walls, while the trophozoites rapidly died in the external environment. If ingested, cysts can withstand the gastric environment but not the trophozoites.

Trophozoites remain in the colon in many cases of asymptomatic carriers and producing or passing cysts in their stools. In some cases, trophozoites invade the intestinal lumen or extraintestinal sites such liver, brain and lungs. The invasion results in significant diseases or pathologic manifestation. Exposure to faecal matter during sexual intercourse also can be one of the mode of transmission (Centers for Disease Control and Prevention, n.d.).

Life Cycle

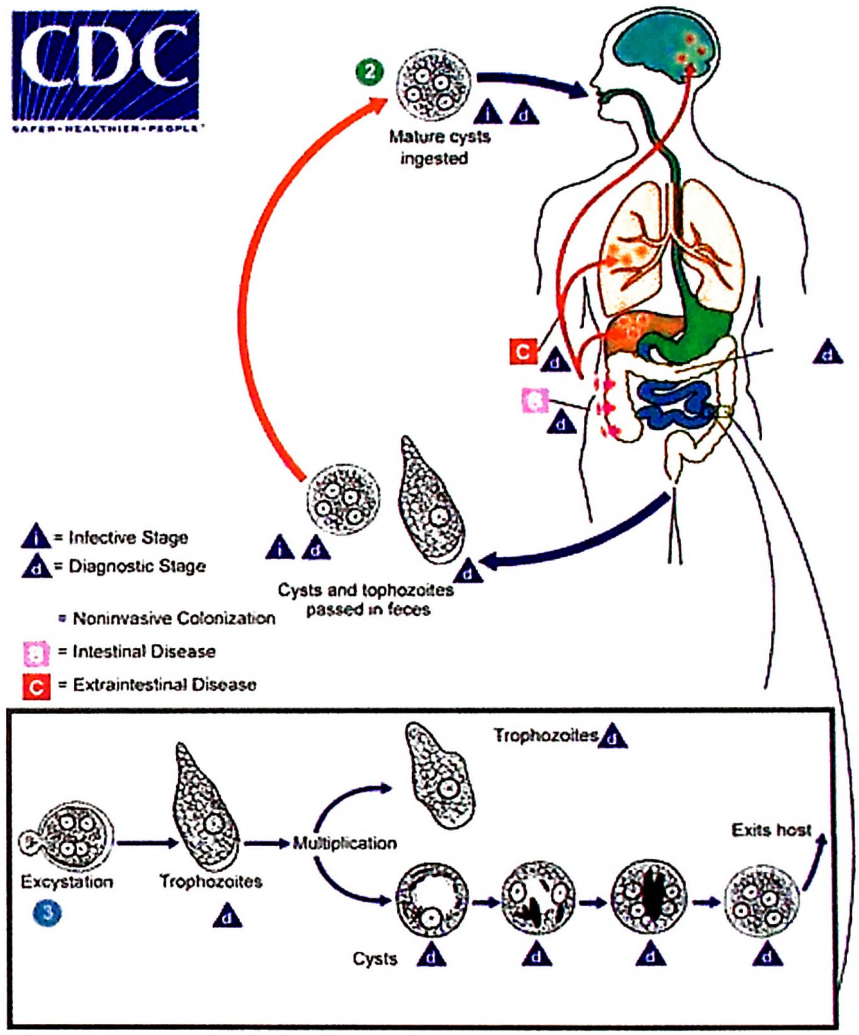

Figure 2.5: Life cycle of *E. histolytica*. Source: www.cdc.gov/dpdx/amebiasis/index.html

2.2. Epidemiology of amoebiasis

Amoebiasis is defined as a disease or an infection caused by protozoan parasite *E. histolytica*. An estimated 1.3 million cases of intestinal amoebiasis were reported in Mexico in 1996. This value might include *E. dispar* infection but it is still consistent with findings by serological technique indicating that about more than 8% of the Mexican had amoebiasis (Pan American Health Organization, 1998; Caballero-Salcedo *et al.*, 1994). One hospital in Hue, Vietnam, a city that has population of 1 million people reported that 1500 cases of ALA occurred in 5 years (Pham *et al.*, 1996). A survey conducted by Abd-Alla & Ravdin (2002) in Egypt stated that 38% of individuals in outpatient clinic which presenting with acute diarrhea had amoebic colitis. In 1993, 2970 cases of amoebiasis were reported in USA. From the cases, about 33% cases were happened among immigrants from Mexico and Central and South America, while about 17% cases were happened among immigrants from Asia or the Pacific Islands (Summary of notifiable diseases, United States, 1994). In one study, 10% of 469 individuals with diarrhoea diagnosed with amoebiasis after travelling to a developing country and in another one study, about 0.3% of 2700 German travellers infected with *E. histolytica* after returning to their country from the tropics (Jelinek *et al.*, 1996; Weinke *et al.*, 1990). These showed that travelling to endemic areas was a possible risk factor to get *E. histolytica* infection and that is why history of travelling is mandotary in diagnosing amoebiasis (Shandera *et al.*, 1998; Barnes *et al.*, 1987; Knobloch & Mannweiler, 1983).

The infection of *E. histolytica* is thought to have no association with gender. A study conducted by Tengku *et al.* (2012) indicated there were no significant differences in the prevalence of *E. histolytica* infection between genders. A study on distribution of *E.*

histolytica and *E. dispar* in northern Philippines showed no significant difference between genders in *E. histolytica* infection (Rivera *et al.*, 1998). Usually, hospital-based studies that show predominance of *E. histolytica* infection in certain genders (Tengku *et al.*, 2012). A study about evaluation of intestinal parasites in a training hospital in Turkey showed the prevalence of *E. histolytica* infection in males was 67%, while 33% in females among patients (Ozyurt *et al.*, 2007). Ejaz *et al.* (2011) reported a significantly high prevalence rate of *E. histolytica* infection in females (31.5%) compared with males (19.6%) in a hospital-based study in Pakistan.

Foodborne and waterborne diseases such as protozoan parasitic infections are still a public health concern in Malaysia, but it is well controlled and only localized in certain areas and groups such the aborigines and the remote area (Ministry of Health Malaysia, 2008). Farhana *et al.* (2009) reported a ten year (1999 – 2008) retrospective study of amoebiasis in University Malaya Medical Centre (UMMC), Kuala Lumpur which indicated that 12 (35%) cases were amoebic dysentery and other 22 (65%) cases were ALA. From the 34 cases, 29 cases were Malaysian and the other 5 cases were detected among foreigners. Molecular epidemiology of amoebiasis showed 3.2% (16/500) of Malaysian aborigines were infected with *E. histolytica* (Tengku *et al.*, 2012). Fever, chills and rigors (76%) were the most common clinical presentation, followed by diarrhoea (59%), right hypochondrium pain (50%), hepatomegaly (47%) and jaundice (20%).

2.3. Pathogenesis of amoebiasis

Abdominal pain, diarrhoea, nausea, fever, cramps, vomiting and flatulence are the major symptoms of amoebiasis (Ngui *et al.*, 2012; Noor Azian *et al.*, 2006). Up to 90% of the amoebiasis cases are asymptomatic whereas the other 10% show a wide spectrum of clinical symptoms such as dysentery and abscesses of the liver or other organs (Farhana *et al.*, 2009).

Amoebiasis begins when trophozoites of *E. histolytica* adhere to colonic epithelial cells through the galactose/N-acetylgalactosamine specific lectin, which is a complex of a disulphide-linked 170 kDa and 35/31 kDa subunits, and an associated 150 kDa protein (Mann, 2002). Amoebic trophozoites become immovable within minutes when they touched human colonic cells and their cytoplasmic granules, structures and nucleus are then lost (Ravdin *et al.*, 1980; Berninghausen & Leippe, 1997).

E. histolytica trophozoites also can kill mammalian cells by apoptosis (Ragland *et al.*, 1994). Host-cell apoptosis was detected in mice with ALA and intestinal amoebiasis. This suggested that the same fate might happen in human intestinal epithelial or liver cells. Independence of Fas and tumour necrosis factor (TNF) α receptor 1, hepatocyte apoptosis requires activation by caspases (Seydel & Stanley, 1998; Huston *et al.*, 2000; Yan & Stanley, 2001). The trophozoites, independent of their cytolytic activity cause the alterations in intestinal permeability. A rapid decrease in transepithelial resistance that come first with any morphological disruption of monolayer integrity is a result of trophozoites added to the apical surface of polarized Caco-2 cell line. It might be associated with disruption of tight junction proteins (Li *et al.*, 1994; Leroy *et al.*, 2000).

Hallmark of amoebic colitis is the invasion of amoebic trophozoites through the mucosa and invade into the submucosal tissues. The classic flask-shaped ulcer of amoebiasis is a result from the lateral extension through submucosal tissues (Stanley, 2003). Contact between amoebic trophozoites and the extracellular matrix protein fibronectin might be the trigger factor for invasion to happen. Signalling cascades within the parasite cause actin rearrangements that results in alteration of adherence and motility (Meza, 2000). In invasion and inflammation of the gut, *E. histolytica* cysteine proteinases are directly implicated (Stanley, 2003). The cysteine proteinases digest extracellular matrix proteins which might aid in invasion of trophozoites into and within the submucosal tissues (Que & Reed, 2000). Interleukin-1-mediated inflammation also might be amplified with the cysteine proteinases by resembling the action of human interleukin-1 converting enzyme, then cleave the precursor interleukin-1 into its active mature form (Zhang *et al.*, 2000).

Studies showed that intestinal epithelial cells sense *E. histolytica* infection and they produce interleukin-1B, interleukin-8 and cyclooxygenase (COX)-2 in response which might have several effects including attraction of neutrophils and macrophages to the site of amoebic invasion (Seydel *et al.*, 1997; Stenson *et al.*, 2001). Neutrophils can be lysed by *E. histolytica* and this property might be the cause of mediators releasing that can cause diarrhoea and tissue damage. This explains why neutrophils are seen less in human biopsy samples (Jarumilinta & Kradolfer, 1964; Seydel *et al.*, 1998; Prathap & Gilman, 1970).

E. histolytica trophozoites encounter additional host defences including complement system as they invade through the mucosa and submucosal tissues, and even into portal circulation. Highly glycosylated and phosphorylated lipophosphoglycan/proteophosphoglycan glycosyl phosphatidylinositol-anchored

molecules cover the trophozoites (Stanley *et al.*, 1992; Bhattacharya *et al.*, 1992; Marinets *et al.*, 1997; Moody-Haupt *et al.*, 2000). These molecules act as physical barriers to complement system. The trophozoites can be protected from lysis because of the structure within the 170 kDa heavy chain of the amoebic Gal/GalNac lectin. This structure has a region with antigenic crossreactivity with CD59, a membrane inhibitor of the C5b-9 attack complex in human red blood cells (Braga *et al.*, 1992). Anaphylatoxins C3a and C5a, as well as human IgA and IgG are cleaved and inactivated by *E. histolytica* cysteine proteinase to give further defence against host immune system (Kelsall & Ravdin, 1993; Reed *et al.*, 1989).

Unique abscesses which are well circumscribed regions of dead hepatocytes, liquefied cells and cellular debris are created when *E. histolytica* trophozoites reaching the liver (Stanley, 2003). Mediators released by lysis process of neutrophils by the trophozoites lead to hepatocyte death and broaden damage to hepatocytes (Burchard *et al.*, 1993; Tsutsumi, 1988). Small numbers of amoebas proportionate to the size of abscess from amoebic liver abscesses from people suggest that *E. histolytica* can kill hepatocytes without direct contact (Stanley, 2003). However, roles of interferon-gamma and inducible nitric-oxide synthase (iNOS) in control of amoebic infection and size of liver abscess were found in knockout mice study (Seydel *et al.*, 2000).

2.4. Treatment of amoebiasis

Single agent or a combination of drugs for the extra-luminal parasite might be the treatment for amoebiasis. By consuming metronidazole followed by a luminal agent such as paromomycin, iodoquinol or diloxanide furoate, amoebic colitis could be treated (Powell *et al.*, 1969; Pehrson & Bengtsson, 1984). Fulminant amoebic colitis should be managed conservatively and addition of antibiotic was given to deal with the bowel flora (Monga *et al.*, 1976). In asymptomatic individuals who are infected by *E. histolytica*, luminal agent treatment should be given to eradicate infection because the individual's faeces might contain *E. histoltytica* cysts which are risk to the public (Gathiram & Jackson, 1987; Haque *et al.*, 2001). To aid in amoebic colitis management, radiographic monitoring of the abdomen by CT scan and combine with catheter drainage to obtain suspect fluid might be useful (Stanley, 2003). Amoebicides that acted effectively in both liver tissues and intestinal lumen include nitroimidazole derivatives which are metronidazole, tinidazole and ornidazole (Sharma & Ahuja, 2003). Liver abscess can be cured with one dose of treatment but the patients should be treated with luminal agent to eliminate intestinal colonization. Surgical drainage of uncomplicated ALA should be averted (Akgun et *al.*, 1999). Single-agent therapy with metronidazole gives effective results. Over 90% of ALA cases are overcome effectively with nitroimidazoles including metronidazole with dose of 40 mg/kg/day in divided dosage (Sharma & Ahuja, 2003).

2.5. Amoebic colitis

Amoebic colitis is an example of invasive intestinal amoebiasis. The other manifestations of intestinal amoebiasis are appendicitis, amoeboma and amoebic stricture (Sharma & Ahuja, 2003). Bloody diarrhoea, abdominal pain and abdominal tenderness are the features of amoebic colitis patients, besides weight loss and anorexia could be present (Adams & MacLeod, 1977a; Lewis & Antia, 1969). Profuse and watery diarrhoea might be noted, while multiple small volume mucoid stools were common (Stanley, 2003). Stools are almost regularly positive with haem because *E. histolytica* trophozoites invade the colonic mucosa even when no blood is seen in the specimen (Adams & MacLeod, 1977b). Leucocytes could be present in the stool samples, in fact, pus could be visible in severe cases, but the numbers are normally not as high as in shigellosis (Harris, 1898; Speelman *et al.*, 1984). Fulminant amoebic colitis, profuse bloody diarrhoea, fever, leukocytosis, widespread abdominal pain often with peritoneal signs and extensive implication of the colon were the features that amoebic colitis patients occasionally developed (Lewis & Antia, 1969; Aristazabal *et al.*, 1991; Ellyson *et al.*, 1986; Bank *et al.*, 1971; Takahashi *et al.*, 1997). Risk factors for fulminant amoebic colitis are pregnant women, immunosuppressed individuals and especially patients receiving corticosteroids, while diabetes and alcohol consumption have also been reported as an association to the fulminant disease (Takahashi *et al.*, 1997; Abioye, 1973). Patients with severe amoebic colitis usually appear very ill and characterized with dysenteric stools, diffuse abdominal pain, high fever and severe dehydration (Takahashi *et al.*, 1997).

2.6. Amoebic liver abscess

ALA is the most common extraintestinal manifestation of invasive amoebiasis. ALA emerges from hematogenous spreads, maybe via the portal circulation of *E. histolytica* trophozoites penetrate the colonic mucosa (Stanley, 2003). The trophozoites make a space-occupying with inflammation at the patient's liver. Due to a large number of patients who are presented with variants and better understandings of the pathogenesis and presentation of ALA, the classical description of this disease need to be modified (Sharma & Ahuja, 1997; Sharma *et al.*, 1997; Sharma & Ahuja, 2003). In Malaysia, the number of ALA cases are increasing probably because of better diagnosis methods available (Farhana *et al.*, 2009).

ALA occurrs in males seven to nine times more than in females and the majority are in the age group of 20 – 45 years old (Sharma & Ahuja, 2003). However, in another study on 204 ALA cases in the University Hospital, Kuala Lumpur, Malaysia by Goh *et al.* (1987), majority of the patients were males, Indian and age ranging between 30 – 60 years old. In another ten year (1999 – 2008) retrospective study of amoebiasis in University Malaya Medical Centre (UMMC), Kuala Lumpur found that among 34 cases of amoebiasis, 22 (65%) cases were ALA and predominantly in males and among Chineses (Farhana *et al.*, 2009), while before that, a ten year (1984 – 1994) retrospective study of amoebiasis by Jamaiah & Shekhar (1999) also in UMMC reported that the majority of the cases were among Malays and males. The phenomenon of most cases were among males might be explained by the fact that most young adult males worked and stayed far away from homes and usually they often ate meals at the outside such as at hawker stores where

the food may not be prepared clean enough and/or the foods were exposed to the dust and flies (Farhana *et al.*, 2009).

ALA is classified by duration of illness and severity into acute and chronic. Majority of patients have duration of symptoms less than 2 weeks and present with acute illness. Abdominal pain, fever and anorexia were the major features. Abdominal pain is often moderate and positioned to the right upper quadrant or to the epigastrium. Moderate fever is the most common but secondary bacterial infection to the abscess was accompanied by high fever with chills. Also seen in ALA are cough with or without sputum and pleuritic chest pain (Sharma & Ahuja, 2003). About one-third of patients presented with jaundice. Large abscess or multiple abscess, or abscess positioned at the porta hepatis resulted in severe icterus (Data *et al.*, 1973). Up to 80% of patients were reported to have tender hepatomegaly (Sharma & Ahuja, 2003). The most common laboratory findings are leukocytosis without eosinophilia, mild anaemia, raised concentrations of alkaline phosphatase and high value of erythrocyte sedimentation rate (Thompson *et al.*, 1985; Abuabara *et al.*, 1982; Shandera *et al.*, 1998; Barnes *et al.*, 1987; Adams & MacLeod, 1977b).

ALA commonly occurs in the right lobe of the liver and is single abscess (30 - 70%). But, sometime it is the variants that cause alarm to the clinicians. Multiple abscesses, left lobe abscesses, abscesses presenting as comprehensive lesion, and abscesses rupturing into viscera are the uncommon presentations. Patients that have multiple liver abscesses presented with fever, toxaemia, deep jaundice and encephalopathy counted about 15% of patients (Sharma & Ahuja, 2003). About 35% of patients had left lobe abscess and half from them have association with right lobe and the other half have single left lobe abscess

(Sharma *et al.*, 1984). Inferior vena cava obstruction or hepatic outflow obstruction might be due to the ALA located at the back of right lobe (Sharma & Sarin, 1990). Extension of the abscess might result in leakage of the abscess into the pleural cavity with empyema thoracis. Up to 7% of cases had perforation into the peritoneal cavity followed by intra-abdominal extension were usually associated with shock and generalized peritonitis (Sharma & Ahuja, 2003).

2.7. Orang Asli serum samples

Ngui *et al.* (2012) conducted a study of differentiating *E. histolytica, E. dispar* and *E. moshkovskii* using nested polymerase chain reaction (PCR) in rural communities (including aborigines) in Malaysia. They reported 75% (39/52) were positive for *E. histolytica* infections, followed by 30.8% (18/52) *E. dispar* infection and 5.8% (3/52) *E. moshkovskii* infection. They observed that the communities lived in overcrowding areas, have poor environmental sanitation, low levels of education and poor provision of safe water. Rivers located near to the villages were the main source of water for daily needs such as drinking, cooking, bathing and washing clothes. The aborigines also defecated in bushes and/or at nearby river at the back of their houses. These were the risk factors for *E. histolytica* infection.

Noor Azian *et al.* (2006) reported there were 13.2% of *E. histolytica* infections and 5.6% of *E.dispar* infections among the aborigines in Cameron Highland, Pahang. A study on parasitic infection in rural communities in Sabah showed that 21% of the people were infected with *E. histolytica/E. dispar* (Noor Aza *et al.*, 2003). Tengku *et al.* (2012)

conducted a study based on molecular epidemiology of amoebiasis in Malaysia and highlighted the different risk factors of *E. histolytica* and *E. dispar* infections among the aborigine communities. In this study, the prevalence of *E. dispar* was 13.4%, *E. histolytica* was 3.2% and *E. moshkovskii* was only 1.0%. They found three significant risk factors among the aborigines for getting *E. histolytica* infection which were not washing hands after playing with soil or after gardening, indiscriminate defecation in the river or bush and having close contact with domestic animals. In their study population, 75% of the parents had a low level of education. Most of the parents did odd jobs and had a poor income. About 28.6% of them still used nearby river water as the main water source for domestic needs and 38.4% still defecated in river and bushes. About half of them (56.4%) kept domestic animals such as dogs, cats and poultry. The aborigines lived closely with these animals which they were left to roam freely in the villages. Indeed, these animals also slept and defecated indoors. These factors probably make the Orang Asli or aborigines vulnerable to *E. histolytica* infection and other intestinal parasitic infections.

2.8. Diagnosis of amoebiasis

E. histolytica infection is routinely diagnosed with microscopy technique, which involves wet preparation, concentration and permanently stained smear. Fresh or fixed stool samples are examined for cysts and trophozoites. However, cysts and trophozoites of *E. histolytica* cannot be distinguished morphologically with *E. dispar* and *E. moshkovskii* through this method. The presence of ingested red blood cell (RBC) in trophozoites that should be examined within 1 hour of stool collection will facilitate the identification of *E.*

histolytica but only patient with acute dysentery will have RBC in the trophozoites; however asymptomatic passer will only have cysts in their stool (Ngui *et al.*, 2012; Tengku & Norhayati, 2011). Haque *et al.* (1998) reported that microscopy should not be used to diagnose amoebic colitis due to its insensitivity and incapability to distinguish pathogenic *E. histolytica* from non-pathogenic *E. dispar* that could lead to false positive interpretation. Because of that, microscopy technique is unsuitable as a screening test. Besides that, if microscopy is the sole diagnostic method, shigellosis or campylobacter will probably be misdiagnosed as amoebic colitis (Stanley, 2003).

There are some alternative methods in diagnosis of amoebiasis such as serological test for antigen detection or antibody detection. Gal/GalNAc lectin antigen was detected in sera of 75% ALA patients in South Africa (Abd-Alla *et al.*, 1993). A commercial kit developed by TechLab (Blacksburg, Va.) named TechLab *E. histolytica* II test was reported to detect Gal/GalNAc lectin in serum about 96% of ALA patients who did not receive any treatment prior the diagnostic test, but the ability to detect Gal/GalNAc lectin decreased to only 15% positive among the ALA patients after received treatment with metronidazole. In this study, they found that the kit detected antilectin antibodies in 84% of ALA patients that had received treatment, while only 56.5% of ALA patients antilectin antibodies detected before receive the treatment (Haque *et al.*, 2000). Zeehaida *et al.* (2008) reported TechLab *E. histolytica* II antigen detection was not useful for serodiagnosis of ALA in Hospital Universiti Sains Malaysia (HUSM). They compared the standard test at HUSM which was indirect haemagglutination (IHA) with the TechLab *E. histolytica* II ELISA. From 58 patients, 72.4% (42) were positive with IHA, while only 8.6% (5) were positive by the ELISA. Salles *et al.* (2003) reported that IHA was a highly sensitive assay which being

positive in 90% to 100% of ALA patients. IHA was used to detect anti-amoebic antibody in serum of patients or suspected patients. Despite of the highly sensitivity, the interpretation of IHA was quite difficult in endemic areas where the background levels antibody could be high (Zeehaida *et al.*, 2008). Cheaper but valid and reliable in-house diagnostic assay which is comparable to IHA should be developed because IHA commercial kit is rather costly and not suitable for routine screening of invasive amoebiasis in low-resource endemic areas (Tan *et al.*, 2013).

The most popular assay in diagnostic laboratories around the world was ELISA and has been used to diagnose symptomatic amoebiasis after fecal examination (Tengku & Norhayati, 2011). ELISA is a simple, sensitive and reliable quantitative assay (Tan *et al.*, 2013). ELISA could be easily performed in laboratory and could be used as supporting diagnosis of ALA and evaluation of intestinal and extraintestinal amoebiasis where amoebiasis was suspected without detection of *E. histolytica* in faeces (Rosenblatt *et al.*, 1995). In a study by Hira *et al.* (2001), they used microtiter ELISA which was developed by LMD Laboratories Inc. (Carlsbad, CA) to detect antibodies against *E. histolytica*. This study showed 97.9% sensitivity and 94.8% specificity for detection of *E. histolytica* antibodies in ALA patients.

There are many studies of *Entamoeba* prevalence using molecular technique. The first report on successful use of PCR in studying epidemiology of *Entamoeba* infection was by Acuna-Soto *et al.* (1993). DNA extracted from faeces was used in the study. Haque *et al.* (1998) compared nested PCR, isoenzyme analysis and antigen detection test in their study for diagnosis of *E. histolytica* infection. The study indicates the nested PCR was comparable to the other two tests. Use of molecular tools is very crucial in research study

of the infections especially to distinguish pathogenic and non-pathogenic species of the parasites (Noor Azian *et al.*, 2006). Molecular techniques were very assuring tools for epidemiological studies (Ngui *et al.*, 2012). However, use of molecular techniques consumed more times, a bit burdensome and needed more cost (Haque *et al.*, 1998).

2.9. Excretory Secretory Antigen (ESA)

ESA of *E. histolytica* consists of two things which are proteins yield shed from trophozoites during process of active multiplication and metabolites released from trophozoites during incubation in RPMI-C-A medium (Wong *et al.*, 2012). Sengupta *et al.* (2000) studied the role of excretory-secretory products of *E. histolytica* in human amoebiasis ELISA. This study showed 100% positive in diagnosis ALA samples and 100% negative results in diagnosis control samples. Besides that, this study showed 82% positive reaction in acute amoebic dysentery and 45% positive reaction in asymptomatic cyst passers.

Sengupta *et al.* (2000) also did the protein profile of ESA and the result showed bands between 200 kDa and 20 kDa in SDS-PAGE. Common bands at 66 kDa, 56 kDa, 45 kDa and 29 kDa were shown when compared the protein profiles of ESA, crude soluble antigen (CSA) and plasma membrane (PM). In a study by Wong *et al.* (2012), the antigenic bands for ESA ranged between 97.2 kDa to 158 kDa. It was different from study by Sengupta *et al.* (2000). Different ways to prepare the antigen might be the reason of the difference in protein profiles because Wong *et al.* (2012) concentrated the ESA 1000 times instead of 10 times to enrich the protein and use RPMI-C-A medium instead of serum- and

vitamin-mix free TYI-S-33 medium. Two significant antigenic proteins of ESA are the 152 kDa which identified as *E. histolytica* lectin protein and 110 kDa which identified as *E. histolytica* pyruvate phosphate dikinase (Wong *et al.*, 2012).

In another study of other parasite, Choi *et al.* (2003) reported that ESA was better than crude antigen for serodiagnosis of clonorchiansis, an infection of *Clonorchis sinensis*, by using ELISA. In their study, ESA was found to have 92.5% sensitivity and 93.1% specificity, while crude antigen was found to have 88.2% sensitivity and 87.8% specificity. Cho *et al.* (2000) also reported that ESA was a good antigen for serodiagnosis of paragonimiasis, an infection of *Paragominus westermani*.

In a nut shell, ESA of *E. histolytica* could be a better antigen for serodiagnosis for invasive amoebiasis by ELISA.

CHAPTER 3

3. Materials and Methodology

3.1. Materials

3.1.1. Chemical reagents

Chemical reagents used in this study are listed in Table 3.1.

Table 3.1: Chemical reagents

Chemicals	**Manufacturer / Supplier**
Cellognost Amoebiasis:- • 2.5 ml Cellognost Amoeb Reagent • 0.5 ml Cellognost Amoeb Control + • 1.5 ml Cellognost Parasit Control – • 2 X 10 ml Cellognost Amoeb Buffer	Siemens, Marburg, Germany
Acrylamide solution 30%	Bio-Rad, USA
Sodium dodecyl sulphate (SDS)	Vivantis, Malaysia
Ammonium persulfate (APS)	Bio Basic, Canada
Tetramethylethylenediamine (TEMED)	Vivantis, Malaysia
3,3',5,5'- tetramethylbenzedine (TMB) substrate	SIGMA-Aldrich, USA
Phosphate buffered saline	SIGMA-Aldrich, USA
Hydrochloric acid (HCL) fuming 37%	Merck, Germany