ANALYSIS OF EXCRETORY-SECRETORY ANTIGEN OF *Entamoeba histolytica* FOR DETECTION OF AMOEBIC LIVER ABSCESS

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BY

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LIST OF ABBREVIATIONS AND SYMBOLS

<	Less than
%	Percentage
±	More or less
×	Multiply by
≥	More than or equal to
°C	Degree Celsius
µg	Microgram
µL	Microliter
µm	Micrometer
BSA	Bovine serum albumin
c.a.	*circa* - 'Approximately'
cm	Centimeter
CSA	Crude soluble antigen
DNA	Deoxyribonucleic acid
e.g.	*exempli gratia* - 'for example'
et al	*et alii* - 'and others'
etc	*et cetera* - 'and so forth'
g	Gravity
g	Gram
HRP	Horseradish peroxidase
i.e.	*id est* - 'that is'
Ig	Immunoglobulin
IPG	Immobilized pH gradient
kDa	Kilodalton
L	Liter
M	Molar
Mb	Megabase
mg	Milligram
min	Minute
mL	Milliliter
mM	Millimolar
MW	Molecular weight standard
NC	Nitrocellulose
OD	Optical density
PCR	Polymerase Chain Reaction
PES	Polyethersulfone
psi	Pound-force per square inch
TMB	3,3',5,5'-Tetramethylbenzidine
via	'by means of' or 'by way of'
X	Times
β	beta
kVh	Kilovolt per hour
mW	Milliwatt
mA	Milliampere
V	Volt
dH_2O	Distilled water
ECL	Enhanced chemiluminescent
ESA	Excretory-secretory antigen
CBB	Coomassie brilliant blue
RAMA	CBB R250, Acetic acid, Methanol, Ammonium sulphate
HBSS	Hank's balanced salt solution
RPMI	Roswell Park Memorial Institute medium, no 1640
DMEM	Dulbecco's Modified Eagle Medium
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
TYI-S-33	Trypticase-Yeast Extract-Iron and Serum
PBS(A)	Phosphate buffered saline for amoeba
C&A	0.1 % L-cysteine and 0.02 % ascorbic acid
IHA	Indirect Haemagglutination Assay
ELISA	Enzyme-Linked Immunosorbent Assay

ANALISIS ANTIGEN EKSKRETORI-SEKRETORI DARIPADA *Entamoeba histolytica* UNTUK PENGESANAN ABSES HATI AMEBA

ABSTRAK

Amebiasis ialah penyakit protozoa usus yang disebabkan oleh *Entamoeba histolytica*. Ia melibatkan 50 juta orang di seluruh dunia dan menyebabkan 100,000 kes kematian setiap tahun. Abses hati ameba (ALA) merupakan manifestasi klinikal yang paling lazim bagi amebiasis luar usus. Ia boleh menyebabkan kematian jika diagnosis dan rawatan terlewat. Sehingga sekarang, diagnosis untuk ALA masih bersandar kepada hasil keputusan sejarah klinikal, pengimejan radiologi, dan ujian diagnosis makmal. Ujian serologi tetap dijadikan pilihan kerana ia tidak invasif dan senang dijalankan. Namum, ujian serologi yang lazim digunakan, iaitu 'indirect haemagglutination assay' (IHA) dan 'TechLab *E. histolytica* II ELISA' menunjukkan sensitiviti yang rendah di kawasan endemik, disebabkan oleh latar belakang antibodi anti-ameba yang tinggi dalam populasi itu dan penerimaan rawatan terdahulu oleh pesakit. Oleh sebab itu, kajian ini bertujuan untuk menganalisis protein antigenik dalam antigen ekskretori-sekretori (ESA) *E. histolytica*, dan mengenalpasti satu penanda biologi yang berpontensi untuk menambah baik ujian serologi bagi ALA.

Satu larutan media tanpa protein yang mengandungi 0.1 % L-cysteine dan 0.02 % asid askorbik serta dapat memelihara ≥ 95 % trofozoit hidup bagi sekurang-kurangnya 8 jam, iaitu DMEM-C&A atau RPMI-C&A telah dioptimum dan diguna untuk penyediaan ESA. Masa pengeraman trofozoit dalam larutan media itu telah dikurangkan kepada 6 jam bagi mengganti masa yang telah digunakan untuk

mengumpul kultur trofozoit berskala besar. Ketumpatan maksimum trofozoit dalam medium ialah 0.8 x 10^6 sel per mL. Kemudian, ESA dikumpul melalui proses pemekatan dan penukaran larutan penampan sebelum digunakan dalam analisis. Dalam analisis Western blot ESA yang diuji dengan serum manusia berpenyakit ALA (n = 38), dua antigenik protein menunjuk sensitiviti melebihi 80 %, iaitu protein 152 kDa dan 110 kDa. Spesifisiti kedua-dua protein ini ialah 100 % kerana tiada reaksi ditunjukkan apabila ianya diuji dengan serum manusia yang normal (n = 30) dan jangkitan lain (n = 33), di mana IHAnya adalah negatif. Selain itu, analisis Western blot ESA yang diuji dengan serum daripada hamster berpenyakit ALA (n = 9) menunjukkan protein antigenik 130 kDa, 110 kDa dan 100 kDa juga bereaksi dengan antibodi manusia. Analisis lanjutan dengan 2D-Western blot menunjukkan nilai pI bagi ketiga-tiga protein antigenic ini masing-masing ialah 5.33-5.91, 5.91-6.5 dan 5.91-6.5. Keputusan daripada MALDI-TOF-TOF menunjukan protein 152 kDa ialah lektin *E. histolytica* yang telah dilaporkan sebelum ini dan 110 kDa ialah enzim piruvat fosfat dikinase *E. histolytica*. Protein 130 kDa tidak dapat dikenalpasti walaupun proses pemekatan protein telah dicuba, namun kandungannya dalam ESA sangat rendah. Kajian ini berjaya mengenalpasti satu protein daripada *E. histolytica*, iaitu 110 kDa protein yang berpotensi untuk digunakan dalam diagnosis ALA.

ANALYSIS OF EXCRETORY-SECRETORY ANTIGEN OF *Entamoeba histolytica* FOR DETECTION OF AMOEBIC LIVER ABSCESS

ABSTRACT

Amoebiasis is an enteric protozoan disease caused by *Entamoeba histolytica*. It affects 50 million people worldwide and causes up to 100,000 fatal cases annually. Amoebic liver abscess (ALA) is the most common clinical manifestation of extraintestinal amoebiasis. It can lead to fatal outcome if early diagnosis and treatment are not obtained. To date, diagnosis of ALA is dependent on clinical history, radiological imaging, and laboratory diagnosis. Serodiagnosis is widely used because it is non-invasive and easy to perform. However, the commonly used immuno-haemagglutination assay (IHA) and TechLab *E. histolytica* II ELISA showed low sensitivities in endemic areas due to the high anti-amoebic antibody background in the population and prior treatment of patients. Thus, the aims of this study were to analyse the antigenic proteins of excretory-secretory antigen (ESA) collected from *E. histolytica*, and to identify potential biomarker(s) which could improve the serodiagnosis of ALA.

A protein-free defined medium supplemented with 0.1 % L-cysteine and 0.02 % ascorbic acid which could sustain the ≥ 95 % viability of the anaerobic trophozoite for at least 8 hours *i.e.* DMEM-C&A or RMPI-C&A was optimized and the latter used for ESA preparation. The incubation period of trophozoites in the medium was reduced to 6 hours, in order to compensate the time used for harvesting the mass cultured trophozoites. The maximum trophozoites density in the medium was 0.8 x 10^6 cells per mL. ESA was then collected, concentrated and buffer-exchanged prior

to use. Western blot analysis of ESA probed with human serum samples with ALA (n = 38) revealed two antigenic proteins with sensitivities above 80 %, *i.e.* 152 kDa and 110 kDa. Their specificities were 100 % as they were not recognised by normal human serum samples (n = 30) and other infections (n = 33) which were IHA negative. On the other hand, Western blot analysis of ESA probed with hamster serum samples with ALA (n = 9) revealed 130 kDa, 110 kDa and 100 kDa antigenic proteins which were also seen with human sera. Further 2D-Western blot analysis revealed that the pI values of these three antigenic proteins were 5.33-5.91, 5.91-6.5 and 5.91-6.5, respectively. In the MALDI-TOF-TOF analysis, 152 kDa protein was identified as the *E. histolytica* lectin which has been previously reported, 110 kDa as *E. histolytica* pyruvate phosphate dikinase, while 130 kDa protein could not be identified due to its low amount in ESA despite efforts at concentrating it. Thus, this study has successfully identified that 110 kDa *E. histolytica* protein is potentially useful for diagnosis of ALA.

CHAPTER ONE

INTRODUCTION

1.1 GENERAL

Amoebiasis is an enteric protozoan disease caused by *Entamoeba histolytica* (WHO, 1997). It is a cosmopolitan parasitic disease which affects 40-50 million individuals of the world population and causes up to 100,000 of fatal cases annually (Walsh, 1986). It is the third common cause of death due to parasitic disease after malaria and schistosomiasis. It commonly occurs at places with poor sanitation and low socio-economic conditions. The prevalence rate is higher in tropical and sub-tropical countries such as India, Mexico, and in South East Asia (Widmer and Nettleman, 1991).

The high risk groups for amoebiasis include travelers, male homosexuals and those with poor personal hygiene practice (Lazara Rojas Rivero *et al.*, 2008). The transmission is worldwide due to the ease of world travel (Nari *et al.*, 2008). Faecal oral route is responsible for the transmission through the ingestion of the infective stage cysts. On the other hand, human to human transmission through oral-genital or oral-anal contact also have been reported among homosexuals (Hung, 2007). Among 10 % of the infected humans, only 10 % develops the clinical symptoms; while in the remainder the infection persists as asymptomatic carriers. Among the 10 % symptomatic patients, 90 % may present as intestinal amoebiasis and 10 % may develop into amoebic liver abscess (ALA). ALA is the most common clinical

manifestation of extraintestinal amoebiasis and potentially fatal if early diagnosis and treatment is not sought (Zlobl, 2001).

According to a World Health Organization (WHO) report, the elementary means for eradicating of amoebiasis is basically based on the improvement of the quality of living conditions (*e.g.* clean water and food) and education (*e.g.* hand washing and personal hygiene) in the countries where amoebiasis is prevalent (Salles *et al.*, 2003). This strategy was evidenced by the success of Cuba in eliminating malaria, schistosomiasis, leishmaniasis and Chagas by improving socioeconomic conditions, health, sanitation and water supply (Lazara Rojas Rivero *et al.*, 2008). Similar to Malaysia, the statistics of parasitic disease had shown positive outcomes as the number of cases declined over the years after the implementation of socioeconomic development in the country. Nevertheless, the number of parasitic infections is relatively high in Malaysia as compared to the developed countries. For example, the prevalence of water-borne diseases among the aborigines, 'Orang Asli' is still high (Yusof and Ghani, 2009; Ministry-of-Health-Malaysia, 2006; Mak, 2004). Therefore, continuous improvement of health program and monitoring of parasitic diseases are still needed.

1.2 *Entamoeba histolytica*

The trophozoite form of *E. histolytica* was described by Lösch in 1875 from a case of patient with chronic dysentery, whereby, Quincke and Roos later described its cyst form in 1893 (Marshall *et al.*, 1997). The name *Entamoeba histolytica* for dysentery producing amoeba was named by Schaudinn in 1903. In 1925, Brumpt reported the presence of *E. histolytica*-like non-pathogenic strain *E. dispar* (Ackers, 2002;

Jackson, 1998; Brumpt, 1928). According to taxonomy classification, *E. histolytica* belongs to the kingdom protista, subkingdom protozoa, phylum sarcomastigophora, subphylum sarcodina, class lobosea, order amoebida, family endamoebidae, genus *Entamoeba* and species *E. histolytica*. It is a unicellular eukaryotic organism, containing nucleus and multiply through binary fission. It is also a heterotrophic and pleomorphic organism, in which it needs nutrient supplies from the environment and performs locomotion through pseudopodia movement. It is an endoparasite as it stays inside a host and preys for food like bacteria, red blood cells or food particles in host through formation of pseudopodia. Based on electrophoretic karyotyping *via* pulse gel electrophoresis, it was predicted that this parasite contains 14 chromosomes and with the genome size of *c.a.* 24 Mb (Loftus *et al.*, 2005; Stanley, 2003; Baron, 1996).

The life cycle of *E. histolytica* consisted of cyst and trophozoite, as shown in Figures 1.1 and 1.2. Upon entering into human digestive track, the cyst passes through the stomach and excysts at the distal end of ileum or colon where the quadrinucleate cyst is activated by the intestinal secretions and develops into four motile metacysts or metacystic trophozoites. Next, the motile trophozoites are formed and multiply *via* binary fission in the intestine. Under adverse condition, some of the trophozoites may undergo encystment. During encystment, the nucleus undergoes two division processes to produce a quadrinucleate cyst and may be excreted from the body in stool. The subsequent host may get infected when they ingest food or drink, contaminated with the cyst. Thus far, human and some none human primates are the only reported natural hosts (Rivera *et al.*, 2010; Hankenson *et al.*, 2003; Stanley, 2003).

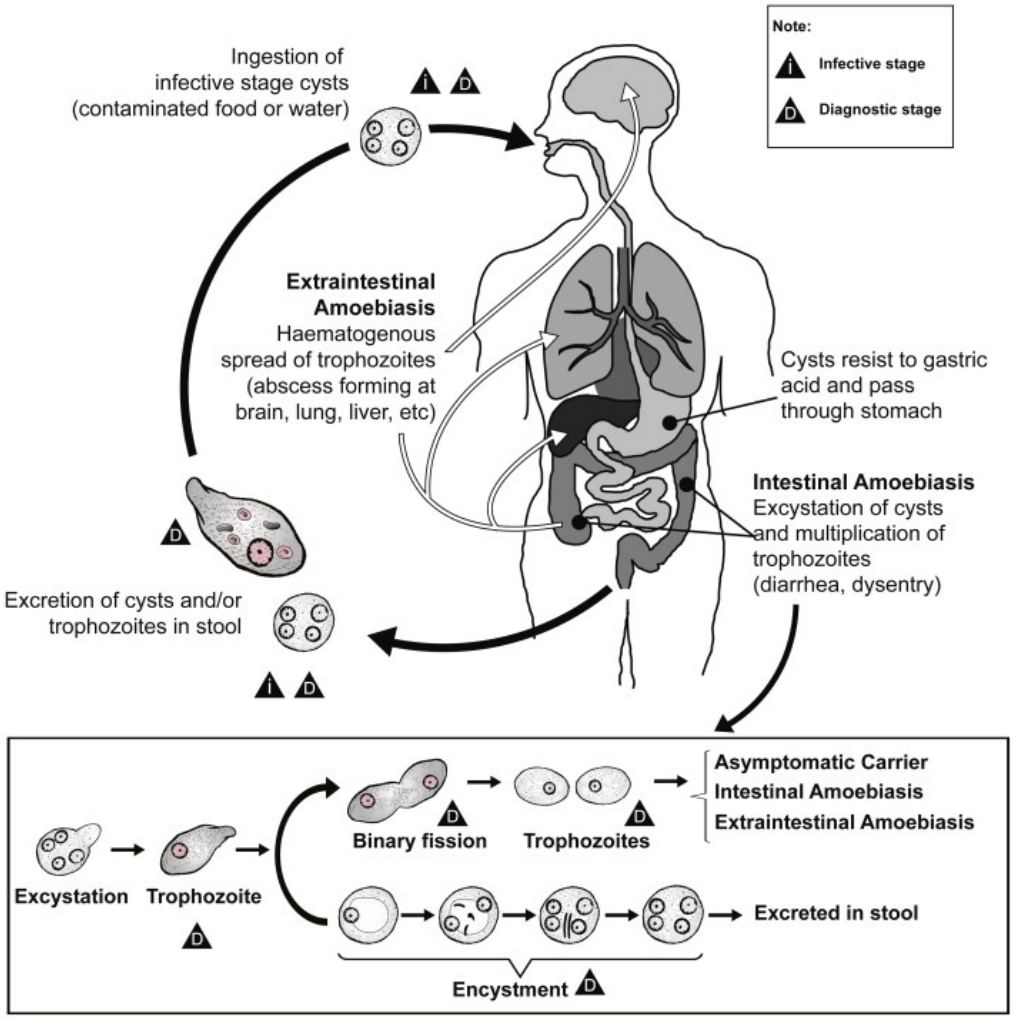

Figure 1.1 Life cycle of *E. histolytica* (CDC, 2010)

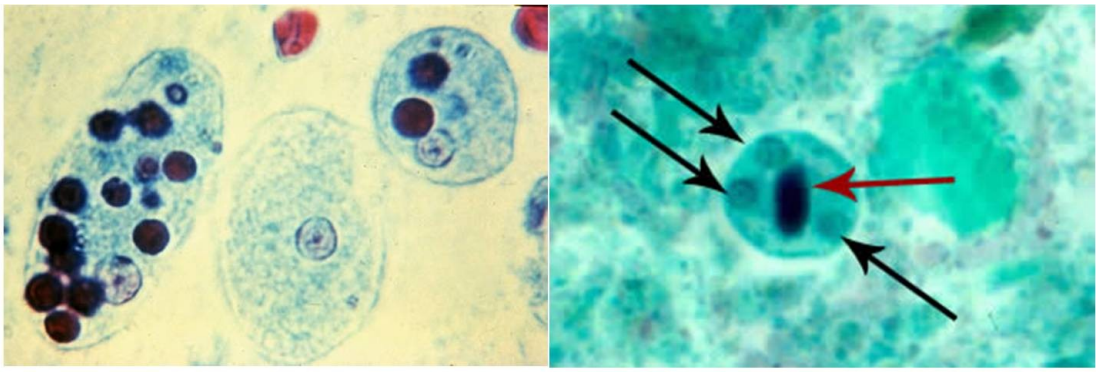

Image source: http://www.dpd.cdc.gov/dpdx/html/imagelibrary/Amebiasis_il.htm

A: Erythrophagocyted trophozoites of *E. histolytica*/*E. dispar*

(Trichrome stained: Erythrocytes appear as dark inclusion bodies. The nuclei with centrally located karyosome and lined by thin layer of uniform peripheral chromatin were stained in blue purple color.)

B: Cyst of *E. histolytica*/*E. dispar*

(Trichrome stained: Three nuclei and a blunt-end chromatoid body were well demonstrated.)

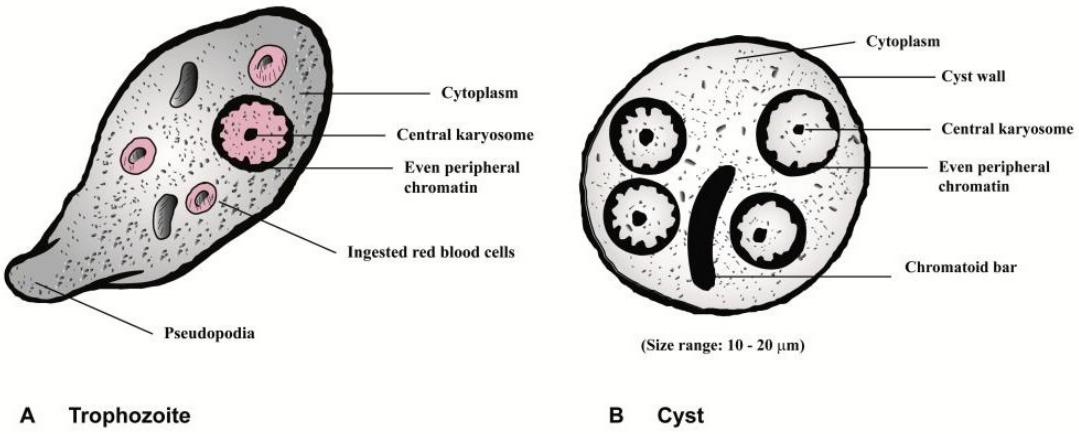

Figure 1.2 Morphological descriptions of *E. histolytica* trophozoite and cyst (CDC, 2010)

The cyst is the infective dormant stage and resistant to environmental stresses (*e.g.* oxygen, gastric acid). It is round in shape, 10-20 μm in diameter, and enclosed by a retractile wall. A mature cyst contains four nuclei. Each nucleus consists of a central karyosome surrounded by peripheral chromatin. Rod-like chromatoid bodies made of the assemblies of glycogen and ribosome may also be present, but they are more common in immature cysts. On the other hand, the trophozoite of *E. histolytica* is actively multiplying and highly motile in human host. Its size is generally 10-60 μm and pleomorphic in shape. It is an anaerobic organism and does not possess mitochondria. The main energy source of trophozoites is acquired from the anaerobic conversion of glucose and pyruvate to ethanol. Red blood cells and/or bacteria are sometimes found in the endoplasm. Under permanent trichrome stain, the nucleus contains a distinctive central karyosome and surrounded by a delicate distinct membrane with beaded chromatin (Lohia, 2003).

1.2.1 Transmission

Generally, *E. histolytica* is transmitted *via* faecal oral route through the ingestion of food and/or water contaminated with cysts, which could come from food handlers, house flies, cockroaches, and the use of human excreta as fertilizers for growing vegetables and fruits (Hankenson *et al.*, 2003). Amoebiasis could also be transmitted among homosexuals *via* oral-genital or oral-anal contact. The life span of trophozoites in stool is rather short. However, the cysts are more robust, resistant to environmental pressure, and able to survive in stool for up to about 12 days. High incidence of amoebiasis occurs among those between 26 – 30 years old as compared to children below 5 years old. The incidents of ALA are higher in males than females with a ratio of 10:1 or higher (Lee *et al.*, 2009; Akgun *et al.*, 1999).

1.2.2 Clinical Manifestations and Pathogenesis

The pathogenesis of amoebiasis is highly dependent on the virulence of trophozoites and host-parasite interaction. The clinical presentations of the individual infected by *E. histolytica* are wide as shown in Table 1.1. The trophozoites may just reside in the infected individual as commensally organism without tissue invasion. If mucosal invasion occurs, the clinical manifestation may vary from local mucosal erosion to mucosal ulceration depending on depth of the invasion. Mucosal erosion may lead to diarrhoea and the severity increased with the area of the affected colon. If the lesions are toward the distal part of the colon, it is more likely the individual will have the symptoms of intestinal amoebiasis, instead of asymptomatic local caecal lesion. When the rectal bleeding occurs, the blood in the faecal specimen may be occult, which is demonstrable only by chemical testing. The individual may also experience abdominal tenderness or cramping pain.

The invasion of trophozoites often started at the intestinal compartment and progress to extraintestinal if the trophozoites break into the blood stream. In the benign phase of intestinal amoebiasis, the individual will experience mild to severe diarrhoea, which is also classified as non-dysenteric colitis. However, when the area and depth of lesion increase, diarrhoea will be replaced by dysenteric stools, which consist largely of mucous and blood without faeces. The affected individual may experience fever, dehydration, and electrolytes imbalance. Occasionally, but less frequently, local invasion of trophozoites will evoke a proliferative granulomatous response at the ulcerative site and turn into pseudo-tumor, known as amoeboma.

Extraintestinal amoebiasis is results from the haematogenous spread of trophozoites to liver, lung, brain or skin. ALA is the most common clinical manifestation which is due to the metastasis of trophozoites to liver through the portal vein. A focal amoebic abscess in the liver would be sufficient to represent the metastasis of trophozoites from intestine to liver. Intestinal amoebiasis symptoms need not be present simultaneously. The size of the abscess may grow bigger with the enlargement of the liver mass in a symptomatic patient. The individual may experience right upper quadrant pain, tenderness of the liver, fever, jaundice and nausea. Fatality may occur if early diagnosis and treatment are not sought.

1.2.3 Host Parasite Interaction

The actual mechanism of host defence against *E. histolytica* remains unclear. Both innate and acquired immunity are reported to be important for prevention of amoebiasis. The first defence of amoebiasis in human is the mucosal layer of intestine, which serves to prevent contact between trophozoites and intestinal epithelial cells. Passive immunity is also contributed by the natural flora present in human intestine. They compete with the trophozoites to adhere to host colonic cells. Often, cell mediated immunity is polarized toward Th1 immune response, in which they limit the extent of invasive amoebiasis and protect the host from recurrent infection following recovery from the disease (Supali *et al.*, 2010; Solaymani-Mohammadi and Petri, 2008).

Table 1.1 Classification of amoebiasis

WHO Clinical Classification of Amoebiasis Infection	Pathophysiologic Mechanism
Asymptomatic infection	Colonization without tissue invasion
Symptomatic infection	Invasive infection
Intestinal amoebiasis	
A. Amoebic dysentery	Fulminant ulcerative intestinal disease
B. Nondysenteric gastroenteritis	Ulcerative intestinal disease
C. Amoeboma	Proliferative intestinal disease
D. Complicated intestinal amoebiasis	Perforation, haemorrhage, fistula
E. Post-amoebic colitis	Mechanism unknown
Extraintestinal amoebiasis	
A. Nonspecific hepatomegaly	Intestinal infection with no demonstrable invasion
B. Acute nonspecific infection	Amoebas in liver but without abscess
C. Amoebic abscess	Focal structural lesion
D. Amoebic abscess complicated	Direct extension to pleura, lung, peritoneum, or pericardium
E. Amoebic cutis	Direct extension to skin
F. Visceral amoebiasis	Metastatic infection of lung, spleen, or brain

On the other hand, the resistance of trophozoites and its virulence factors are the key factors for disease development and progression. The virulence factors of *E. histolytica* included Gal/GalNAc lectin, proteases, and amoebapores. Gal/GalNAc lectin is a surface membrane protein that assists the adherence of trophozoites to the host cell *via* high affinity binding to the O-linked protein, galactose (Gal) and N-acetyl-D-galactosamine (NAcGal) present in the mucosal layer of intestine. This lectin has been shown to contribute to lysis of host cells through contact-dependent killing *via* apoptosis. Following adherence of trophozoites onto the mucosal surface, it secretes proteases that contribute to the erosion of intestinal mucus by disruption of mucin 2 (MUC2) polymerization. These proteases are able to rapidly degrade the mucosal IgA and serum IgG (Garcia-Nieto *et al.*, 2008; Garcia-Zepeda *et al.*, 2007; Tarleton and Petri, 2004; Tran *et al.*, 1998). The trophozoites also secrete an amoebapore, a pore-forming protein which lysed host cells. Besides, the lysed cells like neutrophils and granulocytes also release toxic products that further increase host cell tissue destruction (Leippe and Herbst, 2004). A recent study showed that the trophozoites expressed CD59-like protein on its surface to protect itself against the cytolytic action of the membrane attack complex, by inhibiting the polymerization of the C9 protein, which is responsible for the modification of membrane phospholipid layer (Ventura-Juarez *et al.*, 2009; Zambrano-Villa *et al.*, 2002).

1.2.4 Serological Immune Response of Amoebiasis in Human

Immune responses of human with amoebiasis are complex due to the wide range of its clinical presentation. Upon the invasion of *E. histolytica*, the immune response of human is elicited within seven days (Kaur *et al.*, 2004). As a sign of mucosal invasion, IgA is secreted in the mucosal layer of intestine (Haque *et al.*, 2003). It has

been shown that high level of mucosal-IgA, saliva-IgA and serum-IgG may persist for at least one year after treatment. The levels of these antibodies are higher in patients with ALA as compared to amoebic dysentery cases. These persistent antibodies contribute to the background anti-amoebic antibodies among population in endemic areas. Besides, recurrent infection often occur (Haque and Petri, 2006; Valenzuela *et al.*, 2001; Haque *et al.*, 1997). Higher level of anti-lectin antibodies was reported in patients with amoebic dysentery and ALA, as compared to asymptomatic cysts passer. In ALA cases, high level of IgG1 (60 % of total anti-lectin IgG) was found to be present, which suggested Th2 immune response; while high level of IgG1 (40 % of total anti-lectin IgG) and IgG4 (30 % of total anti-lectin IgG) were found to be present in both patients with intestinal amoebiasis and asymptomatic cysts passer (Kaur *et al.*, 2004). The level of IgM was higher in cases of intestinal amoebiasis, as compared to ALA and asymptomatic cyst passer. High level of anti-amoebic antibodies was reported in endemic areas, but recurrent infections still occur and unremitted in many patients. Therefore the protection roles of these antibodies against *E. histolytica* remain controversial (Tarleton and Petri, 2004; Trissl, 1982).

1.2.5 Other Amoebas

Besides the parasitic amoeba that requires host, there are free living species as well, *i.e. Naegleri*a spp., *Acantamoeba* spp., *Balamuthia mandrillaris* and *Sappinia diploidea* (Schuster and Visvesvara, 2004; Schuster, 2002). Several species of these non-pathogenic amoebas are reported to be found in human body, *i.e. E. polecki, Iodamoeba butschlii, E. coli, E. moshkovskii, E. gingivalis, E. chattoni* and *Endolimax nana* (Zlobl, 2001). Table 1.2 shows the description of the features and

characteristics of the amoebas found in human intestine. Figure 1.3 shows the morphology of the amoebas that could be found in humans stool samples. Since the discovery of the morphologically identical but genetically different *E. dispar*, the result from microscopy is no longer specific for detection of *E. histolytica*. However erythrophagocyted trophozoites are suggested to highly correlate with the presence of *E. histolytica* and invasive disease. *E. dispar* had been classified by WHO as the non-pathogenic strain (WHO, 1997).

1.3 CLINICAL DIAGNOSIS OF AMOEBIASIS

The clinical diagnosis of individual infected with *E. histolytica* could be categorized into three groups based on their clinical manifestations, *i.e.* asymptomatic carrier, intestinal amoebiasis and extraintestinal amoebiasis.

1.3.1 Asymptomatic Carrier

Asymptomatic carriers are those with no signs and symptoms of either intestinal or extraintestinal amoebiasis**,** but with the presence of *E. histolytica* trophozoites or cysts in the intestine or stool. Majority of the affected people falls into this category. They are detected by microscopy examination of trophozoites and/or cysts in the stool. Since microscopy could not distinguish among species of pathogenic/non-pathogenic *Entamoeba*, the application of DNA-based or stool antigen detection test is highly recommended (Baron, 1996).

Table 1.2 Morphologic features and pathogenicity of intestinal amoeba (Tanyuksel and Petri, 2003)

Characteristics	***E. histolytica*, *E. dispar* and *E. moshkovskii*[a]**	***E. hartmanni***	***E. coli***	***E. polecki***	***E. nana***
Trophozoites (size, nucleus, and movement)	15-20 μm; 1 nucleus; actively motile cytoplasmic protrusions, quickly finger shaped pseudopodium	8-10 μm; 1 nucleus; nonsuccessive	20-25 μm; 1 nucleus; slow movement, short and blunt pseudopodium	15-20 μm; 1 nucleus; motility resembles *E. coli*	7-9 μm; 1 nucleus, blunt and hyaline pseudopodium, slow movements
Cysts (size, nucleus)	12-15 μm; mature cyst has 4 nuclei, immature cyst has 1 or 2 nuclei	6-8 μm; mature cyst has 4 nuclei; immature cyst has 1 or 2 nuclei; 2 nucleated cysts very common	15-25 μm; mature cyst has 8 nuclei, rarely 16 or more nuclei	10-15 μm; 1 nucleus, very rarely binucleated or quadrinucleated	6-8 μm; 4 nuclei
Appearance of trophozoites	Stained trophozoites with fine, uniform granules of peripheral chromatin, and small central karyosome in nucleus; ingested RBC (*E. dispar* and *E. moshkovskii* are similar to *E. histolytica* trophozoites, sometimes ingested RBCs)	Nuclear structure similar to *E. histolytica*; cytoplasm finely granular; ingested bacteria	Nuclear with irregular cluster of peripheral chromatin; large, irregular, eccentric karyosome	Nucleus with minute central karyosome, with fine granules of peripheral chromatin, finely granular cytoplasm; ingested bacteria	Nucleus with large karyosome; no peripheral chromatin
Appearance of cysts	Typical nuclear structure is uniform size in having both karyosome and peripheral chromatin, chromatoidal bars with squared or rounded ends	Typical nuclear structure, chromatoidal bars with rounded or squared ends	Typical nuclear structure, sliver-shaped or irregular chromatoidals	Mononucleated; large central karyosome; chromatoid bars with pointed or angular ends, inclusion masses	Chromatin, 4 nuclei with large karyosomes and no peripheral chromatin
Pathogenicity	Only *E. histolytica* is pathogenic (*E. dispar* and *E. moshkovskii* are nonpathogenic)	Nonpathogenic	Nonpathogenic	Nonpathogenic	Nonpathogenic

[a]*E. moshkovskii* is present in free-living protozoa.

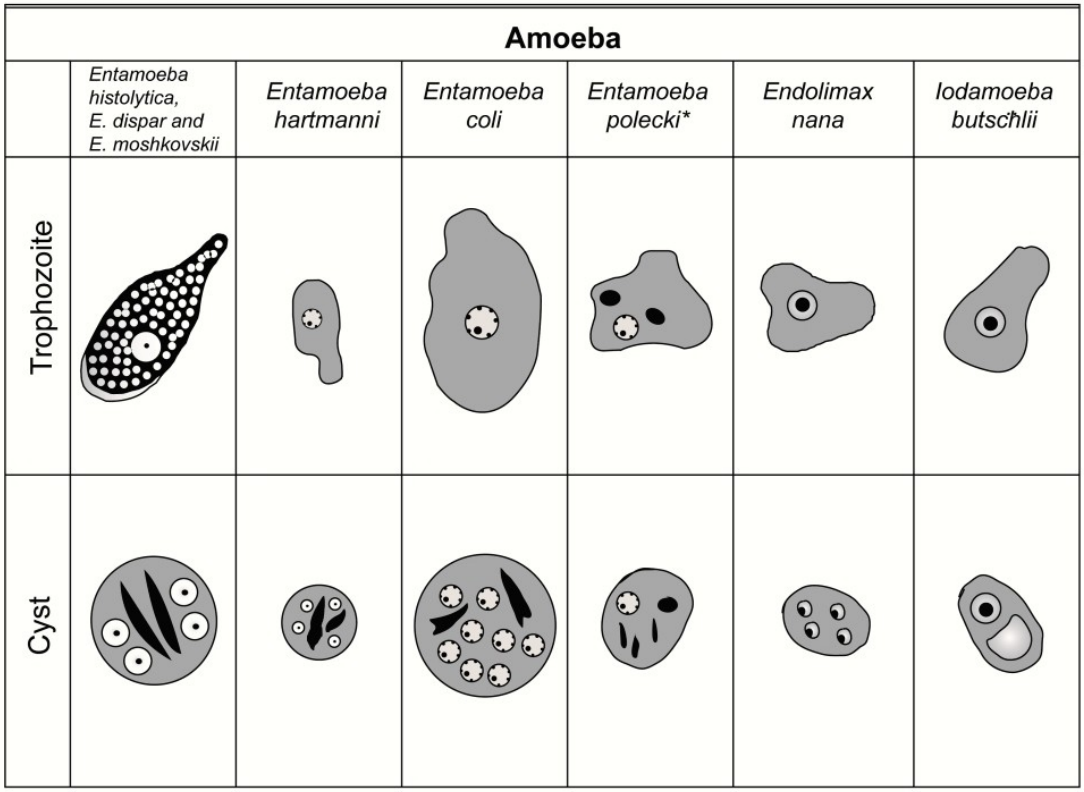

Figure 1.3 Amoebas found in stool specimens of humans (Baron, 1996)

1.3.2 Intestinal Amoebiasis

Intestinal amoebiasis may present as amoebic colitis or amoebic dysentery. In cases suspected of intestinal amoebiasis, stool samples for three consecutive days are collected and sent for microscopy examination. Negative result from single samples does not rule out the possibility of amoebic infection. Polyvinyl alcohol (PVA) fixative or Schaudinn's fixative should be used during the stool specimen collection to preserve the fragile and rapid deteriorating trophozoites. However, direct wet mount on fresh stool samples are commonly performed in many laboratories to save cost and time, but the sensitivity is low. Therefore concentration techniques such as zinc sulfate flotation technique and formalin ether sedimentation technique should be performed on 'negative' samples to decrease the possibility of false negative result. In many cases, live trophozoites could not be detected *via* concentration technique, as many will deteriorate during the process. Antigen detection tests like Techlab *E. histolytica* II and ProSpecT *Entamoeba histolytica* are more sensitive, but they are not routinely used in most of the developing counties due to the high cost. Figure 1.4 (A) shows the flowchart for clinical diagnosis of intestinal amoebiasis.

The clinical presentation of intestinal amoebiasis may be similar to enteric bacteria disease *e.g.* salmonellosis, shigellosis, enteropathogenic or enterohaemorrhagic *Escherichia coli*, as well as noninfectious inflammatory bowel disease and ischemic colitis. Besides, amoebic colitis can also be confused with the clinical presentation of Crohn's disease. It is important to rule out Crohn's disease prior to treatment with corticosteroid therapy as the treatment will worsen the condition of patient (Baron, 1996).

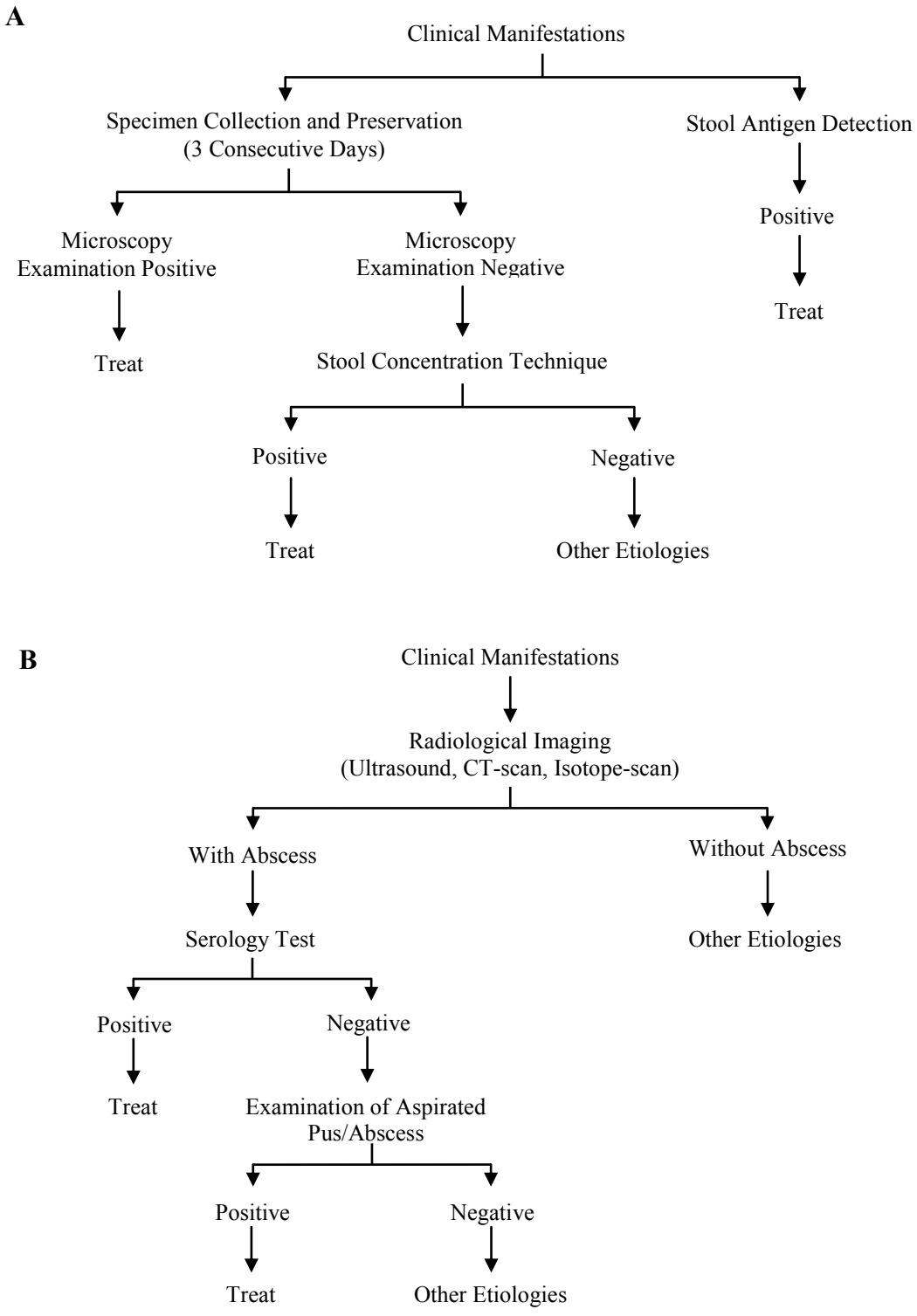

Figure 1.4 Classical flow chart for diagnosis of amoebiasis

A. Intestinal amoebiasis; B. Extraintestinal amoebiasis

1.3.3 Extraintestinal Amoebiasis

The examination for extraintestinal amoebiasis is often started when patient experiences the symptoms of fever, nausea, hepatomegaly and tenderness in the right upper quadrant. Radiological imaging is performed to investigate the presence and size of the abscess. Depending on the physical condition of the patient and size of the abscess; the physician may aspirate the abscess for microscopy examination to check for the presence of live trophozoites, and also to rule out the possibility of liver necrosis. Culture of the abscess is also performed to rule out the possibility of pyogenic liver abscess that are caused by bacteria such as *E. coli*, *Klebsiella* spp., *Staphylococcus* spp. and *Streptococcus* spp. Live trophozoite is rarely found in the microscopy examination of aspirated pus as it is easily disintegrated and tendency of the trophozoites to line the peripheral margin of abscess, instead of the center part (Salles *et al.*, 2003). The trials to harvest the trophozoites at the peripheral margin of abscess are ethically inappropriate, as this may harm the unnecessary normal liver tissue. Furthermore, for extraintestinal amoebiasis cases, the symptoms of intestinal amoebiasis may not be present, and trophozoites and cysts are rarely found in the stool samples. Therefore, many physicians often treat the patient based on the combination of clinical manifestations, serological test and radiological imaging. Figure 1.4 (B) shows the simple flowchart for diagnosis of extraintestinal amoebiasis (Baron, 1996).

1.4 TREATMENT FOR AMOEBIASIS

Upon confirmation of diagnosis, physicians will prescribe drugs for treatment of patient. The drugs for treatment of intestinal and extraintestinal amoebiasis are shown in Tables 1.3 and 1.4. Each of the drugs has its side effects (Abdi *et al.*, 1995).

Physical condition of patient *e.g.* pregnancy will be taken into consideration prior to prescribing the drug. In asymptomatic carrier, once the *E. histolytica* species are confirmed, the patient should be treated to prevent spreading of the infective stage cysts. The drugs of choice included iodoquinol, paromomycin, and diloxanide furoate. The treatment for intestinal amoebiasis is similar to asymptomatic carrier. However, as the disease progress from mild intestinal amoebiasis to severe intestinal amoebiasis such as amoeboma or acute dysentery, the drug of choice will be tinidazole, metronidazole or paromomycin, in which the side effect will be greater.

The most common drug for treatment of extraintestinal amoebiasis is metronidazole. It is a highly lethal drug for anaerobic organism *e.g.* protozoa and bacteria. Besides, it also showed optimum pharmacokinetic features, in which it is easily absorbed in the intestine, has high bioavailability, wide systemic distribution including internal part of the abscess, and with a half-life of 14 hours. The dosage of the drugs that is effective for liver abscess is four times the minimum inhibitory concentration for the trophozoites. The drug can be administrated orally or by intravenous injection. The prescription for oral route is 750 mg of metronidazole, three times daily for 10 days for adult and 50 mg/kg/day for children. Whereby, 500 mg of metronidazole is introduced by intravenous infusion every 8 hours for 5 or 10 days. The common side effects of metronidazole include nausea, vomiting, headache and abdominal discomfort. Metronidazole should not be given to pregnant women during the first trimester of pregnancy and breastfeeding, as the drug is able to cross the placenta and able to enter breast milk. However, if amoebiasis is diagnosed in pregnant women, metronidazole is still the drug of choice but close follow up is needed (Salles *et al.*, 2003; Upcroft and Upcroft, 2001; Zlobl, 2001).

Table 1.3 Drugs treatment for amoebiasis (The-Medical-Letter, 2010)

Clinical Classification	**Drug of Choice**	**Dosage**	
		Adult	**Pediatric**
Asymptomatic Carrier	Iodoquinol	650 mg *PO tid* x 20d	30-40 mg/kg/d (max 2g) *PO* in 3 doses x 20d
	OR Paromomycin	25-35 mg/kg/d *PO* in 3 doses x 7d	25-35 mg/kg/d *PO* in 3 doses x 7d
	OR Diloxanide furoate	500 mg *PO tid* x 10d	20 mg/kg/d *PO* in 3 doses x 10d
Mild to Moderate Intestinal Amoebiasis	Metronidazole	500-750 mg *PO tid* x 7-10d	35-50 mg/kg/d *PO* in 3 doses x 7-10d
	OR Tinidazole	2 g once *PO* daily x 3d	≥ 3yrs: 50 mg/kg/d (max 2g) once *PO* x 3d
	(Either followed by Iodoquinol)	650 mg *PO tid* x 20d	30-40 mg/kg/d (max 2g) *PO* in 3 doses x 20d
	OR Paromomycin	25-35 mg/kg/d *PO* in 3 doses x 7d	25-35 mg/kg/d *PO* in 3 doses x 7d
Severe Intestinal and Extraintestinal Amoebiasis	Metronidazole	750 mg *PO tid* x 7-10d	35-50 mg/kg/d *PO* in 3 doses x 7-10d
	OR Tinidazole	2 g once *PO* daily x 5d	≥ 3yrs: 50 mg/kg/d (max 2g) *PO* in 1 dose x 3d
	OR (Either followed by Iodoquinol)	650 mg *PO tid* x 20d	30-40 mg/kg/d (max 2g) *PO* in 3 doses x 20d
	OR Paromomycin	25-35 mg/kg/d *PO* in 3 doses x 7d	25-35 mg/kg/d *PO* in 3 doses x 7d

Note: *Tid* (thrice a day), d (day), *PO* (by mouth)

Table 1.4 Mechanism and adverse effect of treatment drug for amoebiasis (Stanley, 2003)

Drug	Mechanism	Adverse Effect	Comments
Metronidazole Or Tinidazole	Activated in anaerobic organisms by reduction of the 5-nitro group. Activated compound damages DNA	Metallic taste, nausea, vomiting, diarrhea. (Rarely result in sensory neuropathies, central nervous system toxicity with ataxia, vertigo, seizures and encephalopathy.)	Drug of choice for amoebic colitis and ALA.
Paromomycin	Aminoglycoside (inhibit protein synthesis)	Nausea, vomiting, cramps, diarrhea	Drug of choice for intestinal amoebiasis. It should be administrated to all individuals following completion of metronidazole therapy.
Iodoquinol	unknown	Headache, nausea, vomiting. Optic nerve damage and peripheral neuropathy reported in patient exceeding recommended dosage	Alternative to paromomycin
Diloxanide Furoate	unknown	Flatulence	Alternative to paromomycin

1.5 LABORATORY DIAGNOSIS OF AMOEBIASIS

Conventional diagnosis for amoebiasis is based on microscopy and isoenzyme analysis. With the advancement of technology, there were remarkable developments in molecular biology-based diagnostic tests for detection of *E. histolytica* including enzyme-linked immunosorbent assay (ELISA), indirect haemagglutination assay (IHA) and latex agglutination. With the advent of rapid diagnostic platforms, research on diagnostic kits that enhance point-of-care has also been initiated (Fotedar *et al.*, 2007; Petri and Singh, 1999).

1.5.1 Microscopy

Before molecular techniques were introduced, the diagnosis of amoebiasis is mainly based on clinical syndrome and microscopic examination of stool samples, which posed many problems. First, amoebiasis is often clinically under-diagnosed in developing areas, unless there is history of the patients returning from tropical area. Second, there is poor correlation between patients infected with amoeba and the development of symptomatic amoebiasis, as 90 % of infected individuals present as asymptomatic carriers (Barrett-Connor, 1971).

Besides, before the morphological similarity with the non-pathogenic strain, *E. dispar* was discovered, over-diagnosis and over-treatment were common. The third problem is the poor sensitivity of laboratory methods and low laboratory proficiency. Furthermore, stool examination require fresh samples as well as trained personnel to interpret the result (Mukhopadhyay *et al.*, 2000; Barrett-Connor, 1971). However, microscopic examination of stool samples for the protozoan morphology is still commonly practiced in many parasitology laboratories, especially in

underdeveloped countries. It is mainly applied for diagnosis of intestinal amoebiasis but not extraintestinal amoebiasis, unless intestinal symptoms are present as well. It is difficult or even impossible to microscopically differentiate among all the human intestinal protozoa with similar morphological features, as shown in Figure 1.3 and Table 1.2. Thus, the sensitivity and specificity of microscopy examination for detection of *E. histolytica* in stool are low, due to the possibilities of misdiagnosis with other similar species *i.e. E. dispar* and *E. moshkovskii* (Liang *et al.*, 2009; Haque and Petri, 2006; Tanyuksel and Petri, 2003; WHO, 1997; Haque *et al.*, 1995).

The probable differential diagnosis based on microscopic identification of *E. histolytica* is the presence of trophozoites with red blood cells in dysenteric stool. However, the presence of trophozoites with ingested red blood cells is not frequent in all the intestinal cases. The possibilities of observing trophozoites is higher in loose stool which contained mucous, pus and trace amount of occult blood, whereas cysts could be observed in both formed and loose stools. The fresh stool samples must be processed fast as the trophozoites are rapidly disintegrated. If examination could not be performed immediately, the stools should be preserved in PVA or Schaudinn's fixative (Garcia and Shimizu, 1998).

The microscopy examination of stool samples can be performed directly or after staining. For direct stool examination, wet mount is commonly performed. The trophozoites and cysts can be easily identified, but the nucleus or central karyosome is difficult to see. With Lugol's iodine stain the internal features of the trophozoites and cyst are easily visible. Other stains *e.g.* methylene blue, Giemsa, Wright's and iodine-trichrome may be used for the staining as well, but Wheatley's trichrome

staining and modified iron haematoxylin permanent stain have been suggested for routine used (Fotedar *et al.*, 2007). In Wheatley's trichrome staining, trophozoites in stool samples are stained blue purple, while the background is stained light green. In addition, this stain also displays good chromatin lining and central karyosome of the nucleus. A recent study introduced an easy to perform Eosin Y staining method for trophozoites (*E. histolytica*/*E. dispar*/*E. moshkovskii*) in stool samples. The characteristic features of the amoeba are easily identified. The trophozoites are stained light red in stool samples, while the central karyosome and chromatin materials are stained distinctly dark in colour. This stain is suggested to be suitable for routine diagnosis as it is easy to perform and rapid. However, it is not for long term record keeping, as the stain fades away after one month (Tan *et al.*, 2010).

1.5.2 Biochemical Method: Culture and Isoenzyme Analysis

Culture of *E. histolytica* in artificial media *e.g.* Locke-egg medium, TYSGM-9, and TYI-S-33, followed by isoenzyme analysis has long been performed in many laboratories as the gold standard because it is able to differentiate between the pathogenic and non-pathogenic strains of *Entamoeba* spp. Four of the glycolytic enzymes namely hexokinase, phosphoglucomutase, glucose-6-phosphate isomerase (GPI), and malic enzyme have been applied in the isoenzyme analysis (Razmjou *et al.*, 2006). A total of 24 different zymodemes (isoenzyme analysis patterns) have been established, in which 21 zymodemes are from human isolates (9 patterns for *E. histolytica* and 12 patterns for *E. dispar*) and 3 are from experimentally cultured amoeba strains. Zymodeme is reliable in differentiation of *E. histolytica* and *E. dispar* due to the genetically different hexokinase enzymes between the species. The drawbacks of this method in laboratory diagnosis include being laboratory intensive

and time consuming. This method may need four to ten days to grow the trophozoites to a significant amount prior to starch-gel electrophoresis, and furthermore the culture may not always be successful (Haque and Petri, 2006; Ackers, 2002). Moreover, bacteria or other intestinal protist *e.g. Blastocystis hominis* may overgrow during the culture of trophozoites, and thereby affect the result of the zymodeme. The usage of this technique is now mainly for research purpose or epidemiological study (Fotedar *et al.*, 2007; Gatti *et al.*, 2002).

1.5.3 Antibody Detection

Serological method is commonly used in many laboratories for diagnosis of amoebiasis as the serum samples could be easily obtained and it is relatively less invasive. In endemic area, the detection of antibodies may be difficult due to the persistent high background antibody titer in the population. It is also usually unable to distinguish recent infection from past infection (Pillai *et al.*, 1999). Thus, serological result could only be as a supporting evidence for physician's diagnosis. In contrast, antibody detection method is effective for invasive amoebiasis cases in developed countries, as most of the individuals have no history of past infection.

In ALA, detection of anti-amoebic antibodies in serum samples was claimed to be ~100 % in sensitivity. Therefore, serological method is still a promising choice for diagnosis, together with other evidences from clinical manifestations and radiological imaging. The available antibody detection tests include ELISA, IHA, counterimmunoelectrophoresis (CIE), amoebic gel diffusion, complement fixation (CF), indirect fluorescent assay and latex agglutination. Nevertheless, the commonly used formats for diagnosis of amoebiasis are ELISA and IHA.