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RECOMBINANT PYRUVATE PHOSPHATE DIKINASE (rPPDK): DEVELOPMENT OF ANTIBODY AND ANTIGEN RAPID DIPSTICK TESTS FOR AMOEBIASIS AND IDENTIFICATION OF POTENTIAL ANTI-AMOEBIC COMPOUNDS

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by

SYAZWAN BIN SAIDIN

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

±	more or less
°C	degree Celcius
µg	microgram
µL	microliter
µM	micromolar
ABTS	2,2'-Azino-d-[3-ethylbenthiazoline sulfonate]
ALA	amoebic liver abscess
AMP	adenosine monophosphate
Anti-ESA	antibody to ESA
Anti-rGal/GalNac lectin	antibody to rGal/GalNac lectin
Anti-rPPDK	antibody to rPPDK
APS	ammonium persulfate
APS	ammonium persulfate
Au-IgG4	colloidal gold conjugate IgG4
bp	base pair
BSA	bovine serum albumin
CDC	Centre for Disease Control
CHO	chinese hamster ovary
CIE	counterimmunoelectrophoresis
CRD	carbohydrate-recognition domain
CS1	cysteine synthase 1
CS3	cysteine synthase 3
CT	computed tomography
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleoside triphosphates
E. coli	*Escherichia coli*
e.g.	*Exempli gratia*-'for example'
ECL	enhanced chemiluminescent
Eh	*Entamoeba histolytica*
EhHK1	*E. histolytica* hexokinase 1
ELISA	enzyme linked immunosorbent assay

ESA	Excretory-secretory antigen
et al	*Et alii*- 'and others'
FDA	Food and Drug Administration
FECT	Formalin-ether concentration technique
g	gravity
g	gram
Gal/GalNAc	galactose and N-acetyl-D-galactosamine
HBA	hydrogen binding acceptor
HBD	hydrogen binding donor
HE	hematoxylin and eosin stain
His	histidine
Hgl	heavy subunit of by galactose-inhibitable lectin protein
HL Y6	haemolysin gene
HRP	horseradish peroxidase
HTS	high-throughput screening
HUSM	Hospital Universiti Sains Malaysia
IC_{50}	concentration of an inhibitor where the response (or binding) is reduced by half
IEC	intestinal epithelial cells
IFA	immunoflouresence assay
IFA	indirect immunofluorescence assay
IFN-γ	interferon gamma
Ig	immunoglobulin
Igl1	intermediate subunit of by galactose-inhibitable lectin protein
IHA	indirect haemagglutination assay
IHA	indirect haemagglutination
INFORMM	Institute for Research in Molecular Medicine
IPG	immobilized pH gradient
IPTG	isopropyl β-D-1-thiogalactopyranoside
kDa	kilodalton
K*i*	inhibition constant
k*m*	Michaelis-Menten constant

kVh	kilovolt per hour
LAMP	loop-mediated isothermal amplification assay
LB	Luria bertani
LDH	lactate dehydrogenase
mAB	monoclonal antibody
Lgl	light subunit of by galactose-inhibitable lectin protein
MALDI-TOF/TOF	matrix-laser desorption/ionization-time of flight/ time of flight
mg	Milligram
MIC	minimal inhibitory concentration
mM	Milimolar
MPPP	Penang City Council
NAD	oxidized nicotinamide adenine dinucleotide
NBT	nitro-blue tetrazolium
NC	nitrocellulose membrane
NCI	National Cancer Institute
NF-κB	nuclear factor kappa
Ni-NTA	nickel-nitrilotriacetic acid
nm	Nanometers
NO	nitric oxide
NTC	non-template control
NTD	neglected tropical diseases
OASS	O-acetyl-L-serine sulfhydrylase
OD	optical density
ORF	open reading frame
PAS	periodic acid–Schiff
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEP	Phosphoenolpyruvate
PFK	phosphofructo-1-kinase
PFOR	pyruvate ferredoxin oxidoreductase
pI	isoelectric point
Pi	inorganic phosphate

pI	isoelectric point
PLA	pyogenic liver abscess
POC	point-of-care
PPDK	pyruvate phosphate dikinase
PPi	pyrophosphate dibasic
PST-1	pancratistatin
PVA	polyvynil alcohol
Pyr	pyruvate
RBC	red blood cells
RC DC™	reducing agent detergent compatible
rDNA	ribosomal DNA
rGal/GalNAc lectin	recombinant galactose and N-acetyl-D-galactosamine lectin
rpm	rotation per minute
rPPDK	recombinant pyruvate phosphate dikinase
SAP	soluble amoebic proteins
Sat-ase	serine acetyltransferase
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SOB	super optimal broth
SREHP	serine-rich *E. histolytica*
TB	terrific broth
TBE	tris-borate-EDTA
TBS	tris buffer saline
TCA	tricarboxylic acid
TEMED	tetramethylethylenediamine
TLR	toll-like receptor
TIM	triosephosphate isomerase
TPP	Triage Parasite Panel
U	enzyme unit
UV	ultra violet
V*max*	maximum rate
WHO	World Health Organization
β-NADH	nicotinamide adenine dinucleotide

REKOMBINAN PIRUVAT FOSFAT DIKINASE (rPPDK): PEMBANGUNAN UJIAN DIPSTIK PANTAS ANTIBODI DAN ANTIGEN BAGI AMEBIASIS DAN PENGENALPASTIAN POTENSI SEBATIAN ANTI-AMEBIK

ABSTRAK

Amebiasis, sejenis penyakit protozoa enterik yang disebabkan oleh *Entamoeba histolytica*, merupakan satu masalah kesihatan awam di kebanyakan negara membangun. Amebiasis intestinal boleh menyebabkan simptom seperti kolitis dan disenteri; manakala amebiasis ekstraintestinal kebiasaannya menyebabkan abses hepar jangkitan ameba (ALA). Asai pengesanan antigen komersil yang tersedia untuk jangkitan intestinal menunjukkan sensitiviti dan spesifisiti yang berbeza-beza manakala ujian yang tersedia untuk ALA menggunakan ekstrak asli antigen parasit mempunyai isu spesifisiti. Sebelum ini, piruvat fosfat dikinase (PPDK) telah dikenalpasti sebagai penanda untuk mengesan amebiasis ekstraintestinal, maka protein dalam bentuk rekombinan (rPPDK) digunakan dalam kajian ini untuk membangunkan ujian pantas dipstik antibodi untuk ALA. Pencarian ejen anti-amebik sangat penting kerana kesan sampingan metronidazole dan laporan kerintangan terhadapnya. PPDK terlibat dalam laluan utama metabolisme tenaga parasit, maka PPDK merupakan satu sasaran yang berpotensi untuk pembangunan ejen anti-amebik baharu. Oleh itu, kajian ini juga bertujuan untuk menyiasat aktiviti anti-amebik secara in vitro daripada sebatian berpotensi dengan menggunakan *E. histolytica* HM-1: IMSS. Gen PPDK telah ditempah untuk diklonkan ke dalam vektor pengekspresan pET28, ditransformasikan ke dalam *E. coli* BL21 (DE3), diekspreskan dan ditulenkan. Sensitiviti dan spesifisiti diagnostik bagi protein tulen rPPDK dinilai melalui pemblotan Western dengan menggunakan sampel serum individu. Untuk perbandingan, prosedur di atas juga dijalankan untuk menghasilkan rekombinan

galaktosa-dan-N-asetil-D-galaktosamina lektin boleh rencat (rGal/GalNAc lektin). Ujian pemblotan Western menggunakan serum daripada pesakit ALA, individu yang sihat dan penyakit lain yang ditanda dengan menggunakan anti-manusia IgG4-berkonjugat horseradish menunjukkan nilai sensitiviti (93.3%) dan spesifisiti (100%) diagnostik paling tinggi berbanding blot yang menggunakan IgG dan IgG1 sebagai antibodi kedua. rPPDK menunjukkan spesifisiti yang lebih baik jika dibandingkan dengan rGal/GalNAc lektin. Ujian pantas dipstik aliran sisi dengan menggunakan rPPDK telah dibangunkan dan dinilai untuk serodiagnosis ALA menggunakan sampel serum daripada pesakit dan kawalan. Keputusan menunjukkan sensitiviti diagnostik sebanyak 96.7% (n=29/30) dan spesifisiti 100% (n=40/40). Dalam membangunkan ujian pantas pengesan antigen untuk amebiasis intestinal, antibodi poliklonal (PAb) kepada rPPDK, rGal/GalNAc lektin dan antigen perkumuhan-rembesan *E. histolytica* (Eh ESA) telah dihasilkan. Pengesanan terbaik *E. histolytica* dalam sampel tinja ditunjukkan apabila anti-rPPDK PAbs digariskan pada dipstik dan PAb anti-Eh ESA berkonjugat-emas digunakan sebagai reagen pengesan. Prestasi ujian dipstik aliran sisi yang dibangunkan telah dibandingkan dengan ujian komersial TechLab *E. histolytica* II ELISA dan ujian PCR "real-time", menggunakan 70 sampel tinja daripada pesakit dan kawalan. Sepuluh sebatian daripada Institut Kanser Kebangsaan (NCI) telah dipilih daripada saringan maya sebelum ini yang dilakukan ke atas tapak ATP model tiga dimensi PPDK. Dengan menggunakan asai enzim, tujuh sebatian menunjukkan aktiviti rencatan, merangkumi NSC349156 dan NSC228137 yang menunjukkan nilai MIC masing-masing pada 25 µM dan 50 µM. Dengan menggunakan asai penurunan NBT, NSC349156 menunjukkan nilai IC_{50} yang terendah (14.04 µM), diikuti oleh NSC228137 (20.7 µM); manakala IC_{50} bagi metronidazole ditentukan pada nilai 6.15 µM. Selepas 24 jam rawatan, NSC228137

dan NSC349156 masing-masing menunjukkan 37.61% dan 75.73% rencatan pertumbuhan. Kesimpulannya, rPDDK telah berjaya digunakan dalam pembangunan ujian pantas antibodi dan juga ujian pengesan antigen masing-masing untuk diagnosis ekstraintestinal dan intraintestinal amebiasis. Tambahan pula, dua sebatian menunjukkan potensi untuk bertindak sebagai agen anti-amebik.

RECOMBINANT PYRUVATE PHOSPHATE DIKINASE (rPPDK): DEVELOPMENT OF ANTIBODY AND ANTIGEN RAPID DIPSTICK TESTS FOR AMOEBIASIS AND IDENTIFICATION OF POTENTIAL ANTI-AMOEBIC COMPOUNDS

ABSTRACT

Amoebiasis, an enteric protozoan disease caused by *Entamoeba histolytica*, is a public health problem in many developing countries. Intestinal amoebiasis may produce symptoms such as colitis and dysentery; while extraintestinal amoebiasis commonly results in amoebic liver abscess (ALA). The commercial antigen detection tests for intestinal infection vary in their sensitivities and specificities; most of them use native parasite antigen extract which have specificity issues. Thus this study was aimed at improving the laboratory diagnosis of ALA and intestinal amoebiasis. Previously, pyruvate phosphate dikinase (PPDK) had been identified as a marker for detection of extraintestinal amoebiasis, thus the recombinant form of the protein (rPPDK) was used in this study to develop a rapid antibody dipstick test for ALA. Search for new anti-amoebic agents is important due to the side-effects of metronidazole and reports of resistance against it. PPDK is involved in a key pathway in the energy metabolism of the parasite, thus is a promising target for a new anti-amoebic agent. Therefore, the present study was also aimed to investigate *in vitro* anti-amoebic activity of potential compounds using *E. histolytica* HM-1: IMSS. The PPDK gene was custom cloned into pET28a(+) expression vector, transformed into *E. coli* BL21 (DE3), expressed and purified. Diagnostic sensitivity and specificity of the purified rPPDK protein were evaluated by Western blot using individual serum samples. For comparison, the above procedures were also performed to produce recombinant galactose-and-N-acetyl-D-galactosamine

inhibitable lectin (rGal/GalNAc lectin). Western blots using patients and control serum samples probed with anti-human IgG4-HRP showed higher diagnostic sensitivity (93.3%) and specificity (100%) as compared to blots using IgG and IgG1 as secondary antibodies. rPPDK was found to show better specificity when compared to rGal/GalNAc lectin. A rapid dipstick test using rPPDK was developed and evaluated for ALA serodiagnosis. The results showed 96.7% (n=29/30) diagnostic sensitivity and 100% (n=40/40) specificity. For the development of an antigen detection rapid test for intestinal amoebiasis, polyclonal antibody (PAb) against rPPDK, rGal/GalNAc lectin and *E. histolytica* excretory-secretory antigens (Eh ESA) were produced. The best detection of *E. histolytica* in stool samples was shown when anti-rPPDK PAb was lined on the dipstick and gold conjugated anti-Eh ESA PAb was used as the detector reagent. Performance of the developed dipstick test was compared with commercial TechLab *E. histolytica* II ELISA and real-time PCR, using 70 stool samples from patients and controls. Ten compounds from the National Cancer Institute (NCI) were selected from a previous virtual screening performed against ATP site of a three-dimensional PPDK model. Using an enzymatic assay, seven of the compounds showed inhibitory activities, they include NSC349156 and NSC228137 with MIC values of 25 µM and 50 µM respectively. Using NBT reduction assay, NSC349156 showed the lowest IC_{50} (14.04 µM), followed by NSC228137 (20.7 µM); while IC_{50} of metronidazole was 6.15 µM. After 24 hours of treatment, NSC228137 and NSC349156 exhibited 37.61% and 75.73% growth inhibition, respectively. In conclusion, rPPDK was successfully used in this study for development of rapid antibody and antigen detection tests for diagnosis of extraintestinal and intestinal amoebiasis respectively. In addition, two compounds showed potential to serve as anti-amoebic agents.

CHAPTER ONE

INTRODUCTION

1.1 Overview of amoebiasis

Neglected tropical diseases (NTD) are a diverse group of chronic and disabling diseases that are found in very poor areas and affect more than one billion people globally (Hotez *et al*., 2007). Many of the world's NTDs occur in poor populations in twenty countries (Group of 20), which are mostly tropical and subtropical such as Brazil, Mexico, Indonesia, India, Bangladesh and China (Hotez *et al*., 2013). The major parasitic diseases of the NTDs include amoebiasis, malaria, leishmaniasis, Chagas's disease, schistosomiasis, lymphatic filariasis, strongyloidiasis, ascariasis, onchocerciasis and trichuriasis. Amoebiasis (caused by *Entamoeba histolytica*) is estimated to affect 50 million people worldwide and causes up to 100 000 deaths annually. In terms of mortality due to protozoan parasites, it ranks in second place after malaria (Stanley, 2003; Fotedar *et al.*, 2007a). Of these, 90% are asymtomatic carrier, whereas the other 10% of infected individuals show clinical symptoms such as colitis, dysentery and extraintestinal amoebiasis (Fotedar *et al.,* 2007a). The most common clinical manifestation of extraintestinal is amoebic liver abscess (ALA) and a delay in diagnosis and treatment may cause fatality (Zlobl, 2001). The disease is usually found in areas with poor sanitary conditions and low socioeconomic status. The only reservoir is humans, and infection occurs *via* food, water or hands contaminated with cyst-containing faeces. Human to human transmission has also been reported, that is, through oral-genital or oral-anal contact, especially among homosexual men (Hung *et al.*, 2008), as well as travellers from endemic areas, and those with poor personal hygiene (Rivero *et al.*, 2008).

The epidemiology of amoebiasis varies between countries and continents due to ecological, social-culture and economic factors (Blessmann *et al.,* 2002a; Haghighi *et al.,* 2003; Ali *et al.,* 2008; Ximanez *et al.,* 2009). For example, Fotedar *et al,* (2007a) reported the prevalence of *Entamoeba* infection is as high as 50% in endemic areas of Central and South America, Africa and Asia. Hung *et al*. (2008) and Watanabe *et al*. (2011) reported an increasing risk of amoebiasis in Taiwan, Japan and South Korea, especially among homosexual men who practice oral-anal sex. In Southeast Asia, this disease is commonly found in the following communities-aborigines, those living in poor sanitary conditions, rural and urban populations with low socioeconomic status, and immigrants from amoebiasis endemic areas (Mahmud *et al*., 2013).

Many diagnostic tests have been developed for the accurate detection of *E. histolytica* in clinical samples, such as polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) (Packers, 2002). However, some ELISAs do not show good sensitivity and specificity when used in low to medium endemic areas, especially in developing and underdeveloped countries (Zengzhu *et al.,* 1999; Fotedar *et al.*, 2007a; Zeehaida *et al.,* 2008). Thus, there is an urgent need to improve the diagnostic techniques for the detection of intestinal and extraintestinal amoebiasis. Despite the prevalence of amoebiasis, there is still no available vaccine to prevent this deadly disease (Stanley, 2006). However, there have been progresses in research on potential vaccine candidates, the immunization route, and understanding of the protective immune responses against amoebiasis (Parija, 2011).

Nevertheless, amoebiasis is still a big challenge to public health in many regions, especially in the ‘bottom billion’ countries (Stanley, 2003). According to WHO (1997), the eradication of amoebiasis is essentially based on the improvement

of the quality of living conditions and education in the countries where the disease is prevalent (Salles *et al.*, 2003). Therefore, continuous improvement of the health programme, as well as monitoring and mapping the prevalence of amoebiasis is needed to achieve better prevention, diagnosis and treatment of the disease.

1.2 *Entamoeba histolytica*

1.2.1 Taxonomy

Traditionally, *Entamoeba* spp. have been placed in the phylum Sarcodina due to the presence of pseudopodia. However, based on molecular trees, Cavalier-Smith (2004) has classified *Entamoeba* spp. in the phylum of Amoebozoa, class of Archamoebea and are closely related to the slime mold. Humans are colonized by several species of *Entamoeba*, however most of them do not cause disease. *E. histolytica* is a well-known human pathogenic species that causes intestinal and extraintestinal amoebiasis. According to Pritt and Clark (2008), two non-pathogenic species of *Entamoeba* i.e. *E. dispar* and *E. moshkovskii* which show identical morphology but are genetically distinct, are responsible for most of the asymptomatic *Entamoeba* infections in humans. Therefore, the term amoebiasis is generally applied only to the disease caused by *E. histolytica* (WHO, 1997).

1.2.2 The biology of *Entamoeba histolytica*

E. histolytica is a unicellular eukaryotic organism that can be detected under the microscope in two different stages, i.e. cysts and trophozoites. Figure 1.1(a) shows the trophozoite ranges in size from 10-60 µM and is pleomorphic in shape. When isolated from the host, the cytoplasm of the trophozoite often shows ingested red blood cells (RBCs). It moves with the help of a single uroid (pseudopodium) and

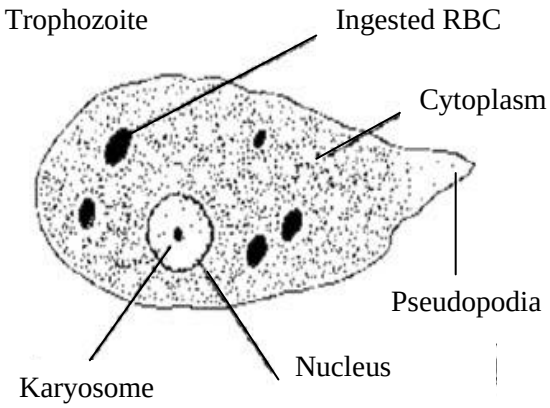
mostly shows a single nucleus. Rough endoplasmic reticulum and Golgi bodies are not visible. Ribosomes are arranged in helices in the cytoplasm and a microtubular cytoskeletal network is not visible. The trophozoite is actively multiplying and highly motile in a human host. The pseudopods produced by an actively moving trophozoite are broad and finger-like, and the parasite generally moves in one direction at a time. *Entamoeba* trophozoites contain proteasomes in their cytoplasm, and these play an important role in cell differentiation and replication (Martínez-Palomo, 1982; Makioka *et al.*, 2002).

The shape of the cyst is round with diameter of 10-20 μM [Figure 1.1(b)]. It is an infective dormant stage and resistant to environmental stresses. A cyst initially has a single nucleus, while 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.

Many of the enzyme metabolites which are produced by *E. histolytica* are believed to be originated from prokaryote, probably obtained via lateral gene transfer from bacteria (Yang *et al.*, 1994; Clark & Roger, 1995; Rosenthal *et al.*, 1997). The cyst cytoplasm has vacuoles with glycogen deposits which can be visible by permanent stains (Lohia, 2013). According to McLaughlin and Aley (1985), *E. histolytica* lacks gluthatione and thus is unable to perform *de novo* purine synthesis, and this differentiates it from higher eukaryotes. In addition, the organization of *E. histolytica* gene is also different from other eukaryotes (Tannich *et al.*, 1992; Clark & Diamond, 1993). The ribosomal DNA (rDNA) episome map has been completely sequenced. The size of the circular DNA of *E. histolytica* is approximately 24.5 kb

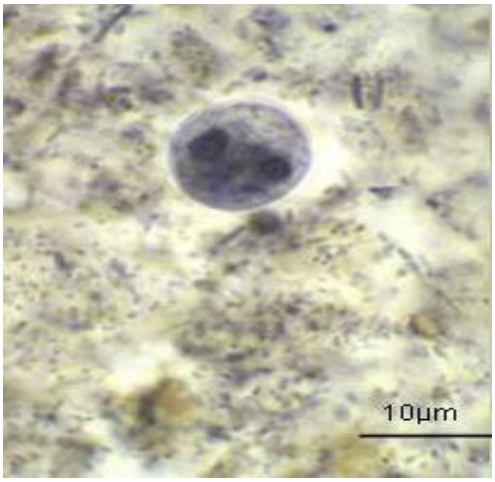
a)

Trophozoite of *E. histolytica* or *E. dispar* stained with heamatoxylin.

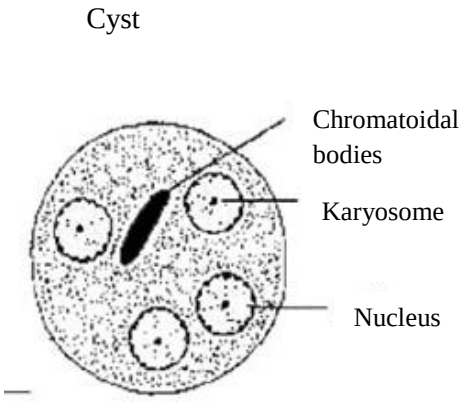


Trophozoite has a single nucleus, which has centrally placed karyosome and uniformly distributed peripheral chromatin.

b)



Cyst of *E. histolytica* or *E. dispar* stained with haematoxylin.



Mature *E. histolytica* or *E. dispar* cyst has four nuclei that characteristically have centrally located karyosome and fine, uniformly distributed peripheral chromatin.

Figure 1.1 Morphological descriptions of *E. histolytica* or *E. dispar* (a) trophozoites and (b) cyst (CDC, 2010).

and has been shown to be useful in genotyping of enteric amoebae (Bhattacharya *et al*., 1989).

E. histolytica, like G*iardia lamblia* is a type I amitochondriate protist, lacking both mitochondria and hydrogenosomes (Martin & Muller, 1998) [type II amitochondriate contains only hydrogenosomes]. These organisms lack features of aerobic eukaryotic metabolism, including the tricarboxylic acid (TCA) cycle and oxidative phosphorylation, and energy generation is primarily by substrate-level phosphorylation. In *E. histolytica*, an extended glycolysis pathway is present, in which pyruvate is converted to acetyl-coA by an enzyme called pyruvate ferredoxin oxidoreductase (PFOR). Subsequently, acetyl-coA can either be converted to acetate with ATP generation, or reduced to ethanol with regeneration of NAD (oxidized nicotinamide adenine dinucleotide) (Muller, 1998; Reeves, 1984; Haanstra *et al.,* 2008).

1.2.3 Life cycle

The *E. histolytica* life cycle is shown in Figure 1.2. The infection usually begins by ingestion of water or food that has been contaminated by *E. histolytica* cysts. Once the cysts reach the distal ileum, the intestinal secretions activate excytation whereby the quadrinucleated cysts develop into four motile trophozoites. Subsequently the amoebas undergo a round of nuclear division, followed by three rounds of cell division by binary fission that produces eight trophozoites. The trophozoites mostly colonize the cecal and sigmoidorectal regions of the large intestine, where they undergo another few rounds of divisions. The motile trophozoites adhere to and invade the cells of the gastrointestinal epithelium.

The invasion of colon is initiated by the parasite adherence and lysis of the colon epithelium, and this process is mediated by galactose and N-acetyl-D-galactosamine (Gal/GalNAc)-specific lectin of the trophozoite (Petri, 2002). Once the intestinal ephitalieum is invaded, trophozoites may spread to other parts of body, and most frequently end up in the liver, causing ALA. The factors that control invasion of the epithelial cells are most probably due to quorum sensing signalled by the amoebic Gal/GalNAc lectin, host innate and acquired immune responses and the interaction of the parasite with the intestinal bacterial flora (Tanyuksel & Petri, 2003). Some of the trophozoites may undergo encystment, in which the nucleus goes through two division processes to produce a quadrinucleate cyst, and may be excreted from the body through the stool. The factor responsible for the induction of encystation is unknown. López-Romero and Villagómez-Castro (1993), and Eichinger (1997) described that encystation is initiated when trophozoites round up into spherical forms, followed by formation of chromatoidal bodies in the cytoplasm. These bodies are ribosomal aggregates and when stained appear as elongated structures with rounded ends. Studies on *E. invadens* suggested that the process of encystation may be triggered by ligation of a surface galactose binding lectin on the surface of the protozoa (Eichinger, 2001; Stanley, 2003). Coppi *et al*, (2002) postulated that trophozoites aggregation in the mucin layer may also be a trigger for the encystation.

The cyst wall is smooth and refractile, and is made up of chitin. The maturation of cyst involves two rounds of nuclear replication, without any cell division. The cyst nucleus is morphologically similar to that of the trophozoite, however it becomes smaller with each division. Cysts can remain alive outside the

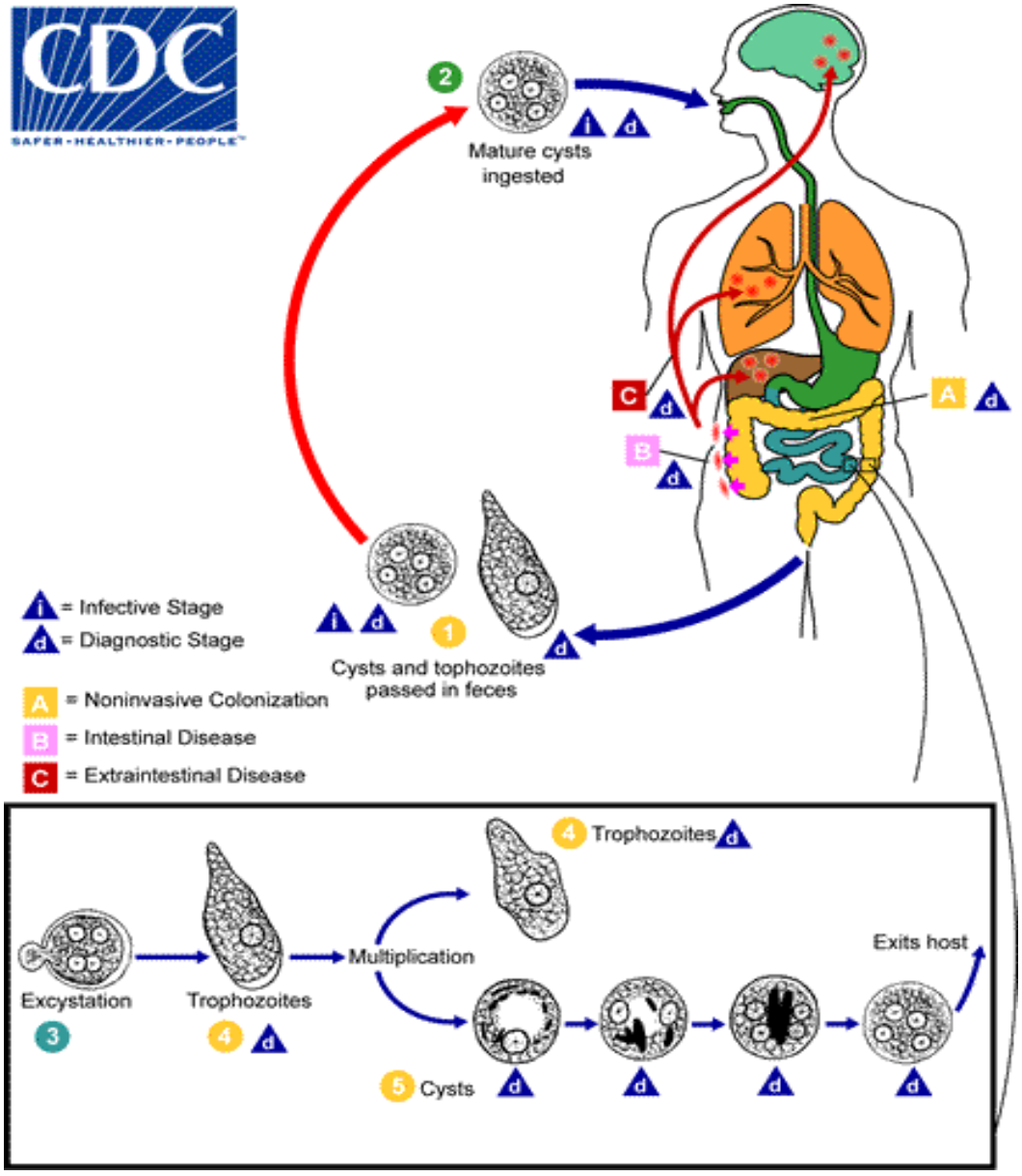


Figure 1.2 Life cycle of *E. histolytica* (CDC, 2010).

host for weeks or months, especially in damp conditions, but are rapidly destroyed at temperatures below -5°C and over 40°C (Fotedar *et al*., 2007a).

The subsequent host may get infected when it ingests food or water contaminated with the cyst. Thus far, human and some non-human primates have been reported as the only natural hosts of *E. histolytica* (Stanley, 2003; Rivera *et al.,* 2010). Other *Entamoeba* spp., i.e. *E. hartmanii*, *E. coli* and *E. polecki*, can be differentiated from *E. histolytica*/*E. dispar*/*E. moshkovskii* by microscopy (Tanyuksel & Petri, 2003; Fotedar *et al.,* 2007a). A detailed description of the microscopic morphologies of the various *Entamoeba* spp. are described in section 1.8.1(a) [Figure 1.5 and Table 1.1] on pages 31-33.

1.3 Mode of transmission

In general, amoebiasis is transmitted via fecal-oral route, through the cysts ingestion from contaminated water or food (Tanyuksel & Petri, 2003). However, the parasite can also be transmitted among homosexual and institutionalized persons via oral–genital or oral–anal contact (Gatti *et al*., 1995; Shelton, 2004; Moran *et al*., 2005). Amoebiasis also spreads through immigrants who reside in endemic areas and travel to other places. Travellers returning from the endemic areas have been reported to have a high risk of infection. For instance, 47 of 469 individuals with diarrhoea were diagnosed as having amoebiasis after travelling to underdeveloped countries (Jelinek *et al*., 1996). Another report showed that 0.3% of 2 700 German travellers had amoebiasis on their return from tropical areas (Weinke *et al*., 1990). According to the Alberta Health and Wellness, Disease Control and Prevention report in year 2003, fewer than 80 cases were reported annually from 1995–2004; the infections were predominantly detected in individuals who have travelled to an underdeveloped area

(http://www.health.alberta.ca/documents/Guidelines-Amoebiasis-2011). Other than the above situations, *E. histolytica* is uncommonly transmitted in industrialized countries, and outbreaks are very rare in such places (Knobloch & Mannweiler, 1983; Salit *et al*., 2009).

In addition, there is ambiguity regarding the zoonotic transmission of the amoeba from dogs, cats, rats, monkeys and probably pigs (Beaver *et al.,* 1984; Krauss *et al.,* 1997). The importance of primates in zoonotic infections was studied by Jackson *et al*. (1990). He investigated whether *E. histolytica* is a true zoonosis using zymodeme analysis. Although *E. histolytica* is commonly associated with animals, there is no report of sporadic transmission between humans and infected animals. In 2005, an amoebiasis outbreak was reported to occur among four chimpanzees kept in captivity at Sandal Kyarimi Park, Nigeria. Based on the laboratory results, cysts and trophozoites of *E. histolytica* were found in the stools of all the chimpanzees during the outbreak. The animals had most likely acquired the infection from the human attendants (Mbya & Nwosu, 2005).

Another study, using a molecular-based method, reported that *E. histolytica* or *E. dispar* infections were detected among wild rats in Malaysia (Lau *et al*., 2014). Together, these studies showed the potential threats of zoonotic transmission as well as the importance of establishing preventive control measures.

1.4 Epidemiological studies on amoebiasis

Epidemiological studies of amoebiasis have been reported since 1925. Many were conducted to investigate the presence of *Entamoeba* spp. in certain regions such as South Africa, Bangladesh, Philippines, Brazil, Egypt and Greece (Haque *et al.*, 1997; Haque *et al.,* 1998a; Braga *et al.,* 1998; Rivera *et al.,* 1998; Gathiram & Jackson,

1987; Abd-Alla & Ravdin, 2002; Haque & Petri, 2006). However, reliable data were only obtained when researchers started to use molecular tools which were able to differentiate between species at the DNA level (Clark & Diamond, 1993; Tannich *et al.,* 1989; Burch *et al.,* 1991). This was because microscopic examination is unable to distinguish among morphologies of *E. histolytica*, *E. dispar* and *E. moshkovskii* (Baron, 1996).

1.4.1 Intestinal amoebiasis

A study by Nath *et al*. (2015) using species specific PCR assay found that the prevalence rate of *E. histolytica* at community health care units and hospitals in a North East Indian population was 13.7%. Ximenez (2009) reported that the incident rate of intestinal amoebiasis in Mexico between 1995-2000 was in range of 1 000 to 5 000 cases per 100 000 inhabitants. An investigation of *Entamoeba* infection in Sudan, using PCR, reported that the highest rate of infection by *E. histolytica*, among microscopic-positive individuals who attended the University of Medical Sciences and Technology (UMST) hospital was 54.1% (Saeed *et al*., 2011). The study also showed that prevalence of co-infection with *E. histolytica* and *E. dispar was* 5.1%, similar to the co-occurrence rate of *E. histolytica* and *E. dispar* in the stool samples in Bangladesh as reported by Haque *et al.* (1998a). Meanwhile, the prevalence of *E. histolytica* infection among preschool children living in Mirpur district of Dhaka, Bangladesh was reported to be 4.2%, as determined by *E. histolytica* II ELISA kit (Haque *et al*. 2006). A survey on the prevalence of intestinal parasites among the expatriates and native Emirati people in UAE showed that protozoan infections (92.2%) were higher than helminthic infections (7.8%). Among the former, *E. histolytica/E. dispar* (71.8%) and *G. lamblia* (17.5%) were the most common

parasites (ElBakri *et al.,* 2013). In Pakistan the prevalence of *E. histolytica/E. dispar,* as detected using formalin-ether concentration technique (FECT) was reported to be 8.6% (Tasawar *et al.,* 2013).

Using PCR assay, Al-Hindi *et al*. (2005) reported higher prevalence rates of *E. histolytica* (69.6%) than *E. dispar* (22.8%) among children in Gaza, Palestine. In a town near Abidjan, Ivory Coast, PCR analysis showed that the ratio of *E. histolytica* to *E. dispar* was 1:46 among microscopically positive samples (Heckendorn *et al*., 2002). A study was conducted by Samie *et al*. (2007) on stool samples in South Africa using nested PCR and ELISA. The results showed that *E. histolytica* was detected in 18.8% and 2.1% of samples from public hospitals and primary schools respectively. Meanwhile *E. dispar* was detected in 25.3 % and 8.5% of samples from public hospitals and primary schools respectively. In a clinical setting in Sweden, Lebbad *et al*. (2005) found that 79.7% (165/207) and 4.8% of patients' stools were PCR-positive for *E. dispar* and *E. histolytica* respectively. In Iran, Mojarad *et al.* (2010) reported that among 3 825 stool samples, 3.5%, 91.4% and 3.5% were PCR-positive for *E. histolytica*, *E. dispar* and *E. moshkovskii,* respectively. Another study conducted among prisoners and primary-school children in Ethiopia showed that real-time PCR detected *E. dispar* in 91.5% (195/213) of microscopy positive samples (Kebede *et al*., 2004). In Australia, among microscopy positive stool samples at St. Vincent's Hospital, PCR analysis showed that 70.8% and 50% were positive for the non-pathogenic *E. dispar* and *E. moshkovskii* respectively (Fotedar *et al*., 2007b).

Meanwhile in Malaysia, real-time PCR on microscopy positive stool samples of Orang Asli (aborigines) showed prevalence rates of 13.2% *E. histolytica* and 9.9% *E. dispar* (Lau *et al*., 2013). On the other hand, a cross sectional study of 500 Orang

Asli stool samples using single-round PCR assay showed a high prevalence of *E. dispar* (13.4%), as compared to *E. histolytica* (3.2%) and *E. moshkovskii* (1%) (Anuar *et al*., 2012). In contrast, using nested PCR, Ngui *et al*. (2012) and Noor Azian *et al*. (2007) reported a high prevalence of 76% and 13.2% *E. histolytica* respectively among the aborigine community. In the year 2004, an amoebiasis outbreak was reported at aborigine villages in the Cameron Highlands, and nested PCR assays were performed on stool samples collected from individuals with diarrhoea. The results showed that *E. histolytica* and *E. dispar* were detected in 13.2% and 5.6% of the stool samples (n=28) respectively (Noor Azian *et al.*, 2006). A study among the Malaysian urban population in Kuala Lumpur demonstrated a low prevalence (0.4%) of *E. histolytica/E. dispar* was reported by Jamaiah and Rohela (2005).

According to Hung *et al*. (2008), in developed countries amoebiasis is more prevalent in older individuals among homosexual men and institutionalized persons. Studies in Japan have shown that *E. histolytica* was more than *E. dispar* among male homosexuals (Takeuchi *et al.,* 1990; Ohnishi *et al.*, 2004). Many researchers from Taiwan, Japan and Korea reported an increasing number of HIV-infected homosexuals who were diagnosed with invasive amoebiasis. Approximately 80% of amoebiasis cases in Japan occurred among homosexuals and most of them were co-infected with HIV and syphilis (Takeuchi *et al*., 1987; Ohnishi *et al*., 2004; Nozaki *et al*., 2006). This finding is in concordance with a study by Hung *et al*. (2005) in Taiwan which showed that HIV-infected individuals were at increased risk for invasive amoebiasis, and showed high frequency of increased antibody titers and *E. histolytica* colonization of the intestine.

After reviewing all the published reports from 1929 to 1997, Acuna-Soto *et al*. (2000) reported that the male to female ratios for invasive intestinal amoebiasis and asymptomatic carriage were 3.2:1 and 1:1, respectively. Most of the asymptomatic carriage infections were due to *E. dispar*. However, according to Rivera *et al*. (1998) who studied 14 communities in the northern Philippines, there was no significant difference in gender distributions of *E. histolytica*. The same observation has also been made in other community-based studies (Okafor & Azubike, 1992; Magambo *et al*., 1998; Hamze *et al*., 2004; Sharma *et al*., 2004; Tasawar *et al*., 2010). Nevertheless, several hospital-based studies recorded gender preference of *E. histolytica* infection. For example, Ozyurt *et al*. (2007) reported *E. histolytica* in 67% males as compared to 33% females among patients at a hospital in Turkey. The study concurred with Ohnishi and Murata (1997), who found that 26 out of 28 males and no females were infected with *E. histolytica* in the east-southeast area of Tokyo, Japan. Conversely, Ozgümüş and Efe (2007) reported that 64% of the attendees of a healthcare centre in Turkey who were infected with *E. histolytica* were females (16/25). Gender comparison of *E. histolytica* infection in Pakistan (hospital-based study) showed higher prevalence rate in females (31.5%) than males (19.6%) (Ejaz *et al*., 2011).

The non-pathogenic species, *E. moshkovskii* has also been reported in several countries though is usually not associated with disease; this include India, Bangladesh, Iran, Australia, Turkey, Malaysia, Tanzania and South Africa (Parija & Khairnar, 2005; Solaymani-Mohammadi *et al*., 2006; Hooshyar *et al*., 2012; Fotedar *et al*., 2008; Anuar *et al*., 2012; Beck *et al*., 2002; Beck *et al*., 2008).

1.4.2 Extraintestinal amoebiasis

With regard to ALA, it is commonly present with an acute and sub-acute history of fever and right upper quadrant pain, although in 20% to 50% of cases showed a more chronic presentation with a protracted history of diarrhoea, weight loss and abdominal pain (Ralph & Kempston, 2012). In developed countries, patients who present with ALA have usually travelled to one of the endemic regions (Salles *et al*., 2003). Knobloch and Manweiler (1983) found that 35% of travellers spent fewer than six weeks in an endemic area before developing ALA. A study conducted in the province of Thua Thien Hue, in Central Vietnam, reported 2 031 cases of ALA between 1990 and 1998, suggesting that the annual incidence of ALA was at least 21 per 100 000 individuals (Blessman *et al*., 2002a; Blessman *et al*., 2002b). The risk of acquiring ALA was higher in summer and was age and gender dependent; whereby 95 % of the cases were adults, and the majority of whom were males (80%). Meanwhile, in a retrospective analysis over a 5 year period, in the medical emergency of a Northern India Hospital, 86 cases of ALA were reported, with a mortality rate of 8.5% (Sharma *et al*., 2010).

Based on a 10-year retrospective study from 1998 to 2008, 38 cases of ALA were reported in many hospitals in Malaysia (Jamaiah & Shektar, 1999). In a recent 15 years (from 1999 to 2014) retrospective study, among 6 867 ill returning Israeli travelers, 53 were diagnosed with all forms of amoebiasis (0.77%), and the rate of ALA among all cases of amoebiasis was 26% (14/53). In addition, twelve of the 14 cases (86%) had an exposure in the Indian subcontinent (Lachish *et al*., 2016). According to Zeehaida *et al.* (2008), 58 cases of suspected ALA infection were clinically diagnosed at the Hospital Universiti Sains Malaysia (HUSM), with 72.4% being seropositive for the presence of anti-*E. histolytica* antibodies. In another study,

approximately 76.7% of the 30 liver abscess cases at HUSM were found positive for the presence of *E. histolytica* DNA in the pus aspirates samples during 2008 and 2009 (Othman *et al.,* 2010). ALA was also reported to affect mostly male patients between the age of 18 and 50, in whom rates were 3 to 20 times higher between populations (De-Bakey & Ochsner, 1951; Abuabara *et al.,* 1982; Katzenstein *et al.,* 1982; Thompson *et al.,* 1985; Barnes *et al.,* 1987; Acuna-Soto *et al.,* 2000). Other than that, ALA was also documented to increase in patients with altered cell-mediated immunity such as HIV infection, which might increase the likelihood of *E. histolytica* infection causing liver disease (Park *et al*., 2007).

1.5 Clinical manifestation of amoebiasis

Amoebiasis produces a varied range of clinical manifestations. Hematologic spread of *E. histolytica* infection can lead to intestinal colonization, colitis as well as extraintestinal amoebiasis. The disease presentations vary in different populations, and depend on factors such as living standard, sanitary condition and nutritional status (Petri & Singh, 1999).

1.5.1 Asymptomatic carrier

The asymptomatic carriers of amoebiasis are individuals with no signs and symptoms of either intestinal or extraintestinal amoebiasis, but with the presence of *E. histolytica* trophozoites or cysts in their intestines or stools. Cysts are usually detected, though trophozoites with ingested red blood cells are rarely seen. Asymptomatic individuals should be treated because they are sources of transmission and the disease can develop and progress into an invasive disease. However, often there is spontaneous resolution of the infection, without disease development (Haque *et al.,* 2001; Blessmann & Tannich, 2002; Blessmann *et al.,* 2006)

1.5.2 Intestinal Amoebiasis

According to Faust *et al.* (1970), within a year, 4-10% of individuals who are asymptomatic may develop the disease. Symptomatic patients usually suffer from intestinal amoebiasis and may present as amoebic colitis or amoebic dysentery. Typical symptoms of amoebic colitis include gradual onset of abnominal pain (over several weeks), tenderness, diarrhoea, fever and bloody stools. The diarrhoea may result in 10 or more bowel movements in a day (Reed, 2000). Commonly, multiple and small volume mucoid stools are observed, however profuse and watery diarrhoea may also be seen (Adams & McLeod, 1977). In addition, bleeding per rectum without diarrhoea may be observed, particularly in children (Ralph & Kempston, 2012). It is surprising that only a small proportion of patients with amoebic colitis have fever (8-38%) (Lewis & Antia, 1970; Adams & McLeod, 1977; Abd-Alla & Ravdin, 2002).

However, it is crucial, albeit difficult to distinguish symptoms of infectious and non-infectious intestinal diseases. Examples of the latter are inflammatory bowel disease, ischemic colitis, diverticulitis, and arteriovenous malformations. These could also be confused with bacterial dysenteries that are common in tropical and subtropical areas such as shigellosis, campylobacteriosis, salmonellosis, and enteropathogenic *Escherichia coli* infection (Haque *et al*., 1997; Sakata *et al.*, 2001). Moreover, the clinical presentation of amoebic colitis can also be confused with Crohn's disease (Baron, 1996).

Occasionally, individuals may develop fulminant amoebic colitis, with profuse bloody diarrhoea, leucocytosis, fever, and severe abdominal pain, and this condition has been reported to result in mortality rate of more than 40% (Stanley, 2003). Toxic megacolon occurs in approximately 0.5% amoebic colitis cases, and is

generally associated with the use of corticosteroids. Since patients with toxic megacolon commonly do not respond to anti-amoebic therapy alone, early recognition and surgical intervention are important. Ameboma can occur as a result of the formation of annular colonic granulation tissue (single or multiple sites) in the cecum or ascending colon. In addition, cutaneous amoebiasis (Mhlanga *et al.*, 1992; Magaña-Garcia & Arista-Viveros, 1993) and rectovaginal fistulas (Lysy *et al.*, 1991) may also be a result of intestinal amoebiasis. Figure 1.3 shows some microscopic images of the above.

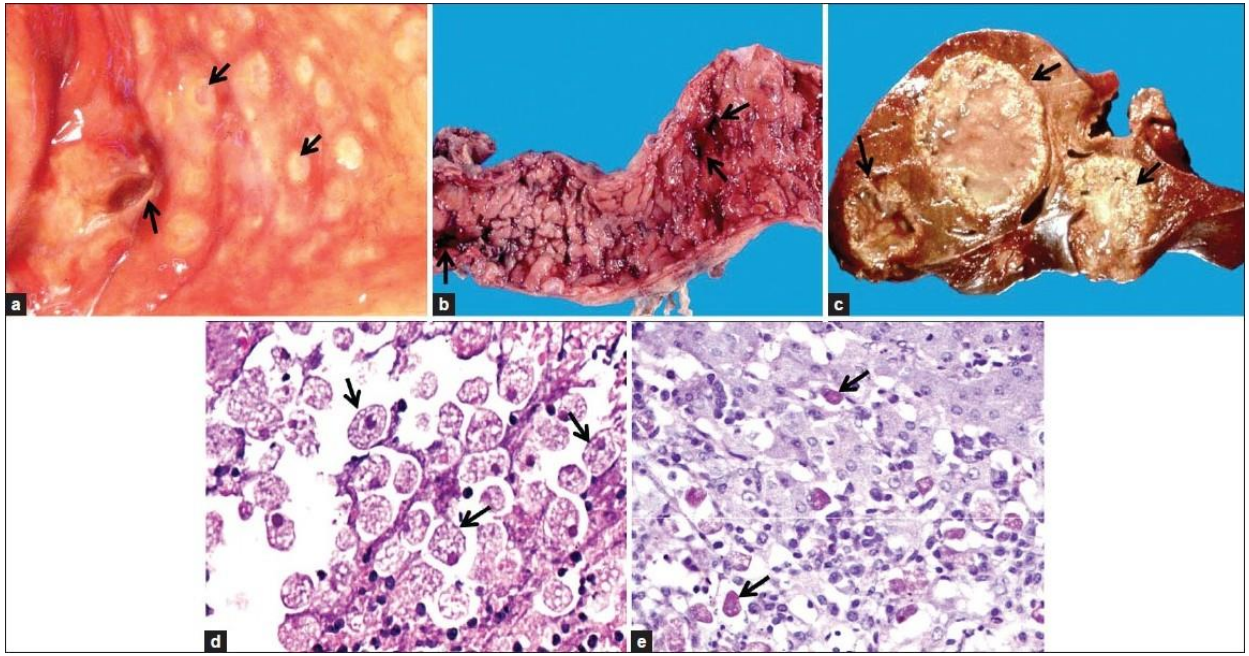


Figure 1.3 Microscopic observations of invasive intestinal amoebiasis, (a) Intestinal flask-shaped ulcers observed through rectosigmoidoscopy examination; arrows indicate the colonic ulcers. (b) Large bowel necropsy specimen from a case of fulminant amoebic colitis; arrows indicate hemorrhagic ulcers and intestinal mucosa necrosis. (c) Necropsy specimen of liver abscesses; arrows indicate the three large abscesses. (d) Intestinal biopsy obtained from the edge of flask-shaped ulcer where large numbers of trophozoites (HE and PAS stained, 60x) are clearly visible. (e) Biopsy obtained from the edge of ALA (HE and PAS stained, 20x); trophozoites, hepatocytes, and the large number of inflammatory cells are indicated by arrows (Ximenez *et al.*,2011).