DEVELOPMENT OF A THERMOSTABILISED MULTIPLEX LAMP-ICT-DNA BIOSENSOR FOR RAPID DETECTION OF *Entamoeba histolytica* AND NONPATHOGENIC *Entamoeba* SPECIES

FOO PHIAW CHONG

UNIVERSITI SAINS MALAYSIA

2017

DEVELOPMENT OF A THERMOSTABILISED MULTIPLEX LAMP-ICT-DNA BIOSENSOR FOR RAPID DETECTION OF *Entamoeba histolytica* AND NONPATHOGENIC *Entamoeba* SPECIES

by

FOO PHIAW CHONG

Thesis submitted in fulfillment of the requirements
for the degree of
Doctor of Philosophy

April 2017

ACKNOWLEDGEMENTS

First and foremost, I would like to express my deepest gratitude to my supervisor, Assoc. Prof. Dr. Lim Boon Huat and co-supervisors, Assoc. Prof. Dr. Chan Yean Yean and Assoc. Prof. Dr. Maizan Mohamed. With their experiences and expertise in this research area, their guidance and supervision assisted me in problem solving and enforced my research progress. Their critical comments have also guided me in the preparation of this thesis. Besides, I would like to extend my gratitude to Dr. Alfonso Olivos Garcia for providing me with the live *E. histolytica* trophozoites and lyophilised *E. dispar* parasites to start this project. Special thanks to Prof. Graham C. Clark for giving me DNA of *E. moshkovskii* needed to evaluate the developed product.

My appreciation also goes out to my colleagues, lab members, administrative staff and laboratory technologists from School of Health Sciences, Department of Medical Microbiology and Parasitology (School of Medical Sciences), Institute for Research in Molecular Medicine (INFORMM) and Faculty of Veterinary Medicine in Universiti Malaysia Kelantan. I would not be able to complete my research project without their help, especially Mr. Amin from INFORMM.

Finally, I am grateful for getting the financial support for my entire Ph.D. endeavour. Thanks to Ministry of Higher Education (MoHE) for the MyBrain15-MyPhD scholarship that covered my tuition fees and living expenses. Not to be forgotten are the three research grants that supported my research project expenses: USM Research University Grant (1001/PPSK/813044), MoHE Long-term Research Grant Scheme (203/PSK/6722002), and MoHE Prototype Development Research Grant Scheme (203/PPSP/6740015).

Last but not least, I would like to extend my appreciation to my family. They have been my strongest motivation and inspiration that kept me focus and complete my study. Despite being the eldest son in the family who was supposed to start working and financially support the family, both of my parents have given me full moral support in continuing my study. In fact, I am lucky to have a loving elder sister who have been taking good care of my parents when I was not with them. As an appreciation, I would like to dedicate this thesis to them and I hope I have made them proud.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

Symbols/ Abbreviations/ Acronyms	Meaning	Symbols/ Abbreviations/ Acronyms	Meaning
°C	Degree Celsius	BSA	Bovine serum albumin
/	Division or “or”	$CaCl_2$	Calcium chloride
=	Equals	CCL	Chromatography control line
≥	Greater than or equal to	CFU	Colony forming unit
≤	Less than or equal to	$C_6H_5O_7Na_3$	Sodium Citrate
µ	Micro	cm	Centimeter
-	Negative or subtraction	DNA	Deoxyribonucleic acid
%	Percentage	dNTPs	Deoxynucleotide triphosphates
±	Plus-minus	EDTA	Ethylenediaminetetraacetic acid
+	Positive or addition		
×	Times or multiplication	ELISA	Enzyme-linked immunosorbent assay
ALA	Amoebic liver abscess	*et al.*	and others
ATCC	American Type Culture Collection	EtBr	Ethidium bromide
Au	Aurum	f	Femto
a.u.	Arbitary unit	F3	Forward outer primer
B3	Backward outer primer	FAM	Fluorescein
BCCM	Belgian Co-ordinated Collections of Microorganism	FIP	Forward inner primer
		FITC	Fluorescein isothiocyanate
BESt	BioHelix express strip	g	Gram
BIP	Backward inner primer	*g*	Relative centrifugal force
BLAST	Basic local alignment search tool	GC	Guanidine-cytosine
bp	Base pair		

Symbols/ Abbreviations/ Acronyms	Meaning	Symbols/ Abbreviations/ Acronyms	Meaning
h	Hour	LB (primer)	Loop backward (primer)
HCl	Hydrochloric acid	LF (primer)	Loop forward (primer)
HRPZ II	Hospital Raja Perempuan Zainab II	LSHTM	London School of Hygiene and Tropical Medicine, London
HUSM	Hospital Universiti Sains Malaysia	LSPR	Localised surface plasmon resonance
IAC	Internal amplification control	LSU-rRNA	Large Subunit ribosomal RNA
IACL	Internal amplification control line	m	Mili
IDT	Integrated DNA Technologies	M	Molar
IHA	Indirect haemagglutination assay	$MgCl_2$	Magnesium chloride
		min	Minute
IMR	Institute for Medical Research	mm	Millimeter
INFORMM	Institute for Research in Molecular Medicine	MTB	*Mycobacterium tuberculosis*
IgG	Immunoglobulin G	MTBC	*Mycobacterium tuberculosis* complex
k	Kilo	n	Nano
KCl	Potassium chloride	*n*	Number of samples
K_2CO_3	Potassium carbonate	NaCl	Sodium chloride
KH_2PO_4	Potassium dihydrogen phosphate	Na_2CO_3	Sodium carbonate
L	Liter	Na_2HPO_4	Disodium hydrogen phosphate
LAMP	Loop-mediated isothermal amplification	NaH_2PO_4	Sodium dihydrogen phosphate
LATE-PCR	Linear-after-the-exponential-PCR	NALF	Nucleic acid lateral flow
LB	Luria Bertani	NALFIA	Nucleic acid lateral flow immunoassay

Symbols/ Abbreviations/ Acronyms	Meaning	Symbols/ Abbreviations/ Acronyms	Meaning
NaN_3	Sodium azide	rRNA	Ribosomal RNA
NaOH	Sodium hydroxide	s	Second
NASBA	Nucleic acid sequence-based amplification	SDS	Sodium dodecyl sulfate
		spp.	Species
NCBI	National Center for Biotechnology Information	*SREHP*	Serine-rich *Entamoeba histolytica* Protein
nm	Nanometer	SSC	Saline-sodium citrate
MW	Molecular weight	SSP2	Sporozoite surface protein-2
OD	Optical density		
		T_a	annealing temperature
p	Pico		
		T1	First target
PB	Phosphate buffer		
		T2	Second target
PBS	Phosphate buffer saline		
		TBE	Tris-borate-EDTA
PCR	Polymerase chain reaction	TL1	First test line
PEG	Polyethylene glycol	TL2	Second test line
PEXT	Primer extension	*TRAP*	Thrombospondin-related anonymous protein
Poly(dA)	Polydeoxyadenylic acid		
		Tris	Tris (hydroxymethyl) aminomethane
Poly(dT)	Polydeoxythymidylic acid		
		Tris-HCl	Tris-hydrochloride
psi	Pounds per square inch		
		U	Unit
PVA	Polyvinylalcohol		
		UPM	Universiti Putra Malaysia
PVP	Polyvinylpyrrolidone		
Q_{10}	Rate of chemical reaction	USM	Universiti Sains Malaysia
RNA	Ribonucleic acid	UV	Ultraviolet
rpm	Revolutions per minute	V	Volt

Symbols/ Abbreviations/ Acronyms	Meaning	Symbols/ Abbreviations/ Acronyms	Meaning
v/v	volume/volume	WHO	World Health Organization
w/v	weight/volume		

PEMBANGUNAN BIOSENSOR TAHAN HABA MULTIPLEKS LAMP-ICT-DNA UNTUK PENGESANAN PANTAS *Entamoeba histolytica* DAN SPESIES-SPESIES *Entamoeba* BUKAN PATOGEN

ABSTRAK

Entamoeba histolytica adalah satu-satunya spesies *Entamoeba* yang berpotensi menyebabkan amebiasis invasif pada manusia. Ameba ini menjangkiti kira-kira 10% daripada populasi dunia dan menyebabkan 100,000 kematian setiap tahun di negara endemik. Asai diagnostik yang baharu diperlukan untuk membezakan *E. histolytica* daripada *Entamoeba dispar* dan *Entamoeba moshkovskii*, iaitu dua spesies *Entamoeba* yang bukan patogen tetapi mempunyai morfologi yang sama. Kaedah mikroskopi tidak dapat membezakan ketiga-tiga *Entamoeba* tersebut. Di samping itu, kaedah ini bukan sahaja memerlukan tenaga mahir, malah ia juga tidak sensitif. Kaedah pengesanan lain seperti asai biokimia, teknik pengesanan antibodi dan antigen mengambil masa yang lama dan/atau memerlukan rangkaian sejuk. Amplifikasi isoterma berpengantara gelung (LAMP) yang hanya memerlukan suhu amplifikasi tunggal berpotensi dijadikan asai point penjagaan. Maka kajian ini bertujuan untuk menformulasi campuran reagen-kering tripleks LAMP untuk mengesan *E. histolytica*, *Entamoeba* spp. dan kawalan amplifikasi dalaman serta membangunkan biosensor NALFIA tanpa penghibridan untuk mengesan amplifikon-amplikon LAMP tersebut. Dua set primer yang mensasarkan *E. histolytica* dan *Entamoeba* spp. telah masing-masing direka bentuk berdasarkan gen *E. histolytica SREHP* dan gen *E. histolytica* subunit besar RNA ribosom. Di samping itu, satu set primer telah direka bentuk dan digabungkan dengan LAMP multipleks untuk berfungsi sebagai kawalan amplifikasi dalaman yang dapat mengesahkan keputusan negatif yang benar. Asai LAMP tripleks telah direka

bentuk untuk menghasilkan amplikon bebenang dua yang dilabel pada dua penghujung dengan pengeraman selama 60 min pada 63°C diikuti dengan suhu penamatan pada 80°C selama 5 min. Amplikon yang dilabel pada dua penghujung ditangkap oleh NALFIA biosensor melalui interaksi pertalian spesifik antara protein dengan hapten pada lokasi pengesanan. Amplikon yang dilabel fluoresein menghasilkan penglabelan hibrid dengan fluoresein antibodi yang dikonjugasikan pada nanopartikel emas. Pengagregatan nanopartikel emas menghasilkan keputusan dalam bentuk garisan merah/merah jambu yang dapat dilihat dengan mata kasar dalam masa 15 min. Pembangunan asai LAMP tripleks bukan sahaja mengurangkan masa amplifikasi, malah dapat mengelakkan penggunaan peralatan yang mahal dan tidak memerlukan kakitangan yang terlatih untuk mengendalikannya. Selain itu, biosensor NALFIA dapat menganalisis amplicon LAMP tanpa penggunaan elektroforesis gel agarosa yang mengambil masa yang agak lama dan menggunakan bahan yang merbahaya. Asai LAMP-NALFIA yang telah dibangunkan dalam bentuk reagen kering bukan sahaja tidak memerlukan rangkaian sejuk, malah ujian diagnostik ini dapat digunakan di tempat rawatan dan lapangan. Had pengesanan asai LAMP-NALFIA ialah 10 trofozoit *E. histolytica* manakala spesifikasinya adalah 100% apabila diuji dengan 71 mikroorganisma lain. Campuran LAMP tripleks yang berasaskan reagen kering dan biosensor NALFIA mempunyai jangka hayat sekurang-kurangnya 181 hari berdasarkan Ujian Kestabilan Haba pada 37°C. Penggabungan dua pelantar asai ini telah berjaya memudahkan pengesanan molekular untuk *E. histolytica* dan spesies-spesies *Entamoeba* yang bukan patogen.

DEVELOPMENT OF A THERMOSTABILISED MULTIPLEX LAMP-ICT-DNA BIOSENSOR FOR RAPID DETECTION OF *Entamoeba histolytica* AND NONPATHOGENIC *Entamoeba* SPECIES

ABSTRACT

Entamoeba histolytica is the only pathogenic *Entamoeba* species that can cause severe invasive intestinal amoebiasis in humans. Approximately 10% of the world population is estimated to be infected, in which 100,000 deaths were reported annually in endemic countries. New diagnostic assays are needed to distinguish *E. histolytica* from its nonpathogenic morphologically identical *Entamoeba* species, *Entamoeba dispar* and *Entamoeba moshkovskii*, as routine microscopy method is labour-intensive, requires highly skilled microscopist, low in sensitivity and unable to distinguish *E. histolytica* from its nonpathogenic species. Other detection methods such as biochemical assays and antibody as well as antigen detection techniques are either time-consuming and/or cold-chain dependent. Loop-mediated isothermal amplification (LAMP) that features single temperature amplification has created an opportunity to develop point-of-care assays. Thus, the present study aimed to formulate a dry-reagent triplex LAMP mix for detection of *E. histolytica*, *Entamoeba* spp. and the incorporated internal amplification control as well as to develop a non-hybridisation-based NALFIA biosensor for detection of the LAMP amplicons. Two sets of specific primers targeting *E. histolytica* and *Entamoeba* spp. were designed based on *E. histolytica* serine-rich protein (*SREHP*) gene and *E. histolytica* large subunit ribosomal RNA gene, respectively. In addition, a set of primers was designed and incorporated into multiplex LAMP to serve as an internal amplification control to rule out false negative result. The triplex LAMP assay was designed to produce

double-labelled double-stranded amplicons with 60 min of incubation at 63°C followed by termination at 80°C for 5 min. These double-labelled amplicons were captured by NALFIA biosensor through specific affinity interaction between capture proteins with haptens on the reaction pad. The fluorescein labelled on each of the amplicons then immobilised fluorescein antibody conjugated gold nanoparticles, and aggregation of gold nanoparticles formed red/pinkish lines which were visualised with naked eye within 15 min. Development of triplex LAMP assay not only reduced the amplification time, it also eliminated the necessity of having thermal cycler which was costly and required trained personnel to operate. Besides, having NALFIA biosensor as amplicons analyser obviated the need for agarose gel electrophoresis which was time-consuming and utilised hazardous chemicals. The triplex LAMP-NALFIA assay that was developed in dry-reagent form was a cold-chain-free and ready-to-use diagnostic test. The detection limit of the assay was 10 *E. histolytica* trophozoites whereas the specificity was 100% when tested with 71 non-*Entamoeba* microorganism. The dry-reagent triplex LAMP mix and NALFIA biosensor had an estimated shelf-life of at least 181 days based on heat stability test conducted at 37°C. Development of the dry-reagent triplex LAMP mix coupled with a NALFIA biosensor had effectively simplified the molecular detection of *E. histolytica* and the nonpathogenic *Entamoeba* species.

CHAPTER 1

INTRODUCTION

1.1 Amoebiasis

Amoebiasis is an enteric disease caused by *Entamoeba histolytica* (Stanley, 2003). This parasitic disease affects approximately 40 to 50 million individuals of world population and causes up to 100,000 deaths annually. It is the third leading cause of death among the parasitic infections after malaria and schistosomiasis (Walsh, 1986; Solaymani-Mohammadi and Petri, 2008; Hegazi *et al.*, 2013). Approximately 10% of the infections are symptomatic ranging from dysentery to amoebic liver abscess (ALA) (Haque *et al.*, 2003).

Amoebiasis is divided into two categories based on its manifestation, namely invasive and non-invasive infections. Most non-invasive category was mostly reported as amoebic dysentery while invasive amoebiasis was mostly associated with ALA. An estimated 10% of intestinal amoebiasis became invasive when amoebae enter into bloodstream and traverse to other organs such as liver where infection could eventually lead to ALA. Among the clinical manifestations of extraintestinal amoebiasis, ALA is the most commonly reported and it could be fatal if treatment is delayed (Zlobl, 2001). Early diagnosis of ALA is challenging as liver abscesses can occur even without patient experiencing prior amoebic diarrhoea. Amoebiasis are commonly reported at low socio-economic and poor sanitation areas. High prevalence rates were found mostly in tropical countries of Central and South America, as well as India (Widmer and Nettleman, 1991). Besides people who live in endemic areas, other high risk groups include travelers and men who practiced oral-anal sex (Hung, 2007; Rivero *et*

al., 2008). Recent study conducted by Wong *et al.* (2016) showed the amoebiasis prevalence was 71% among the Orang Asli adults in peninsular Malaysia.

Several drugs are available for effective treatment of intestinal and extraintestinal amoebiasis in which metronidazole and paromomycin are the most common prescription. However, due to their potential adverse side effects, these drugs should only be given to patients who are confirmed with amoebiasis (WHO, 1997). Physical conditions of patient including pregnancy should also be taken into consideration before prescribing the drug. The common side effects of metronidazole include vomiting, headache, nausea and abdominal discomfort (Upcroft and Upcroft, 2001; Zlobl, 2001; Salles *et al.*, 2003). A summary of commonly prescribed medication for amoebiasis positive patient with its side effect is showed in Table 1.1.

1.2 Pathogenic *Entamoeba* species

E. histolytica is a microaerophilic parasitic protozoan. To date, *E. histolytica* is the only *Entamoeba* species that is responsible for invasive amoebiasis. *E. histolytica* was first described from a case of patient with chronic dysentery in 1875 by Fedor Lösch in St. Petersburg, Russia. Later in 1893, Quincke and Roos described its cyst form followed by Fritz Schaudinn who named the species as *E. histolytica* in 1903 (Maberti, 1994; Momen, 1994; Marshall *et al.*, 1997). Although *E. histolytica* is not the only causative agent for amoebic dysentery, it is described to be the only opportunistic species that potentially cause a wide range of invasive diseases with massive host tissue destruction (Ralston and Petri, 2011). Those diseases include respiratory tract infections, ALA, cerebral and genitourinary amebiasis (Fotedar *et al.*, 2007a). Among them, ALA is the most commonly reported. ALA is associated with a

Table 1.1 Mechanism and adverse effect of treatment drug for amoebiasis

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, diarrhoea. (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, diarrhoea	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

Adopted from (Stanley, 2003)

massive fluid-filled cavity in the parasite infected liver which can be fatal if left untreated (Maltz and Knauer, 1991).

E. histolytica is a unicellular eukaryotic parasite that contain a nucleus and multiply through binary fission. It infects human host through ingestion of its cysts. Excystation takes place in host small intestine which produces 8 trophozoites per cyst, which then colonise the lumen and/or attach to mucus and epithelial cells of large intestine (Petri *et al.*, 2002). It is a heterotrophy endoparasite that stays inside a host and require nutrient supplies from the colonised environment. The pleomorphic characteristic of trophozoite allows it to perform pseudopodia movement and travel to other part of human organ. Human is its primary host where it survives by consuming bacteria, red blood cells or food particles. *E. histolytica* was estimated to contain 14 chromosomes with genome size of 24 mega-basepairs based on electrophoretic karyotyping via pulse gel electrophoresis (Baron, 1996; Stanley, 2003; Loftus *et al.*, 2005).

The life cycle of *E. histolytica* involves two stages, namely infective cyst stage and motile trophozoite stage as shown in Figure 1.1 and Figure 1.2. Despite human being the primary host, other animals can be *E. histolytica* transient or accidental hosts. A quadrinucleate cyst which is activated by the intestinal secretions later developed into four motile metacystic trophozoites. The trophozoites develop and divide through binary fissions and produce 8 trophozoites in the intestine. When the colonised environment is not favorable, encystment takes place to produce a quadrinucleate infective cyst through two division processes, which then passes out along with host stool (Hankenson *et al.*, 2003; Stanley, 2003; Rivera *et al.*, 2010).

E. histolytica cyst can survive and resist environment stress such as presence of gastric acid and oxygen. A cyst typically measures 10 to 20 μm in diameter, consists

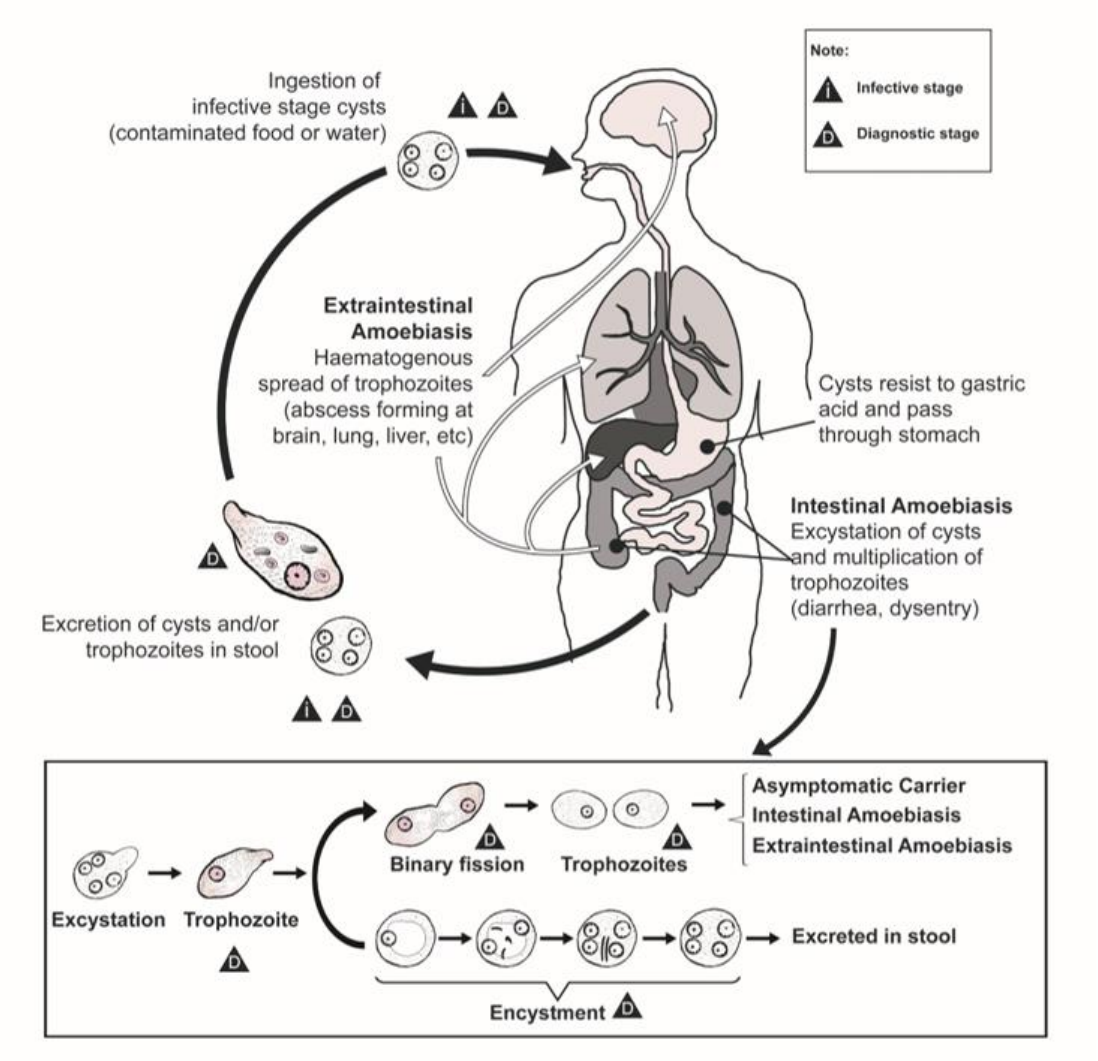

Figure 1.1 Life cycle of *E. histolytica*

Adopted from (CDC, 2010)

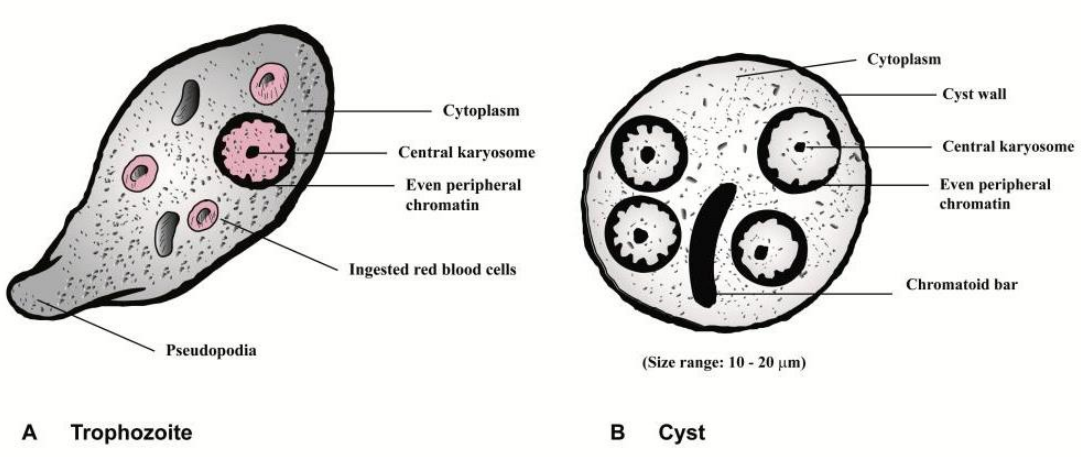

Figure 1.2 Structure of *E. histolytica* trophozoite and cyst

Adopted from (CDC, 2010)

of four nuclei with cigar-shaped or rounded chromatoidal bars made of assemblies of glycogen. However, a vacuole and fewer nuclei may present in immature cyst. Presence of a central karyosome surrounded by peripheral chromatin dots in each nucleus is an important characteristic feature. Similarly, *E. histolytica* trophozoite also possesses such feature (Lohia, 2003). However, it is slightly bigger and measures 10 to 60 µm in diameter and pleomorphic in shape. The trophozoite cytoplasm commonly contains fine ingested red blood cell and/or debris from bacteria. Since, *E. histolytica* is basically anaerobic, it acquires energy through anaerobic conversion of glucose and pyruvate to ethanol.

Generally, *E. histolytica* is transmitted by fecal-oral route due to improper hygiene such as ingestion of cysts via dirty hands or objects (Hankenson *et al.*, 2003). It could also be transmitted through sexual activities that involved oral-anal or oral-genital contact. This may be the reason why more ALA incidents are reported to be higher in males than females with a ratio of 10:1 or higher (Akgun *et al.*, 1999; Lee *et al.*, 2009). Infective cyst is more resistant to environmental pressure, it can survive in stool for up to two weeks whereas only 10% of trophozoites were reported to be viable after 6 h in stool (Tan *et al.*, 2010). The human host invasion of trophozoites often start from the intestinal compartment, then enter the portal vein, and spread haematogenously to the liver, lung, brain or skin.

The pathogenesis of amoebiasis relies on the host-parasite interaction and virulence of trophozoites. Amoebiasis can be categorised into three different groups, which are asymptomatic infection, intestinal amoebiasis, and extraintestinal amoebiasis as shown in Table 1.2. Asymptomatic or mildly symptomatic is the most common manifestation which comprises 90% of the total reported cases based on microscopic examination (Walsh, 1986). Patients with asymptomatic colonisation of

Table 1.2 Classification of amoebiasis

WHO Clinical Classification of Amoebiasis	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. Non-specific hepatomegaly	Intestinal infection with no demonstrable invasion
B. Acute non-specific 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

Adopted from (Sodeman, 1996)

E. histolytica possibly clear their infection without showing any symptom of the disease. Colonised trophozoites may just reside in the infected individual as commensal. However, the opportunistic *E. histolytica* may cause amoebic dysentery or even invasive diseases if left untreated (Gathiram and Jackson, 1987; Haque *et al.*, 2001; Blessmann *et al.*, 2002; Blessmann *et al.*, 2006). Treatment is recommended for asymptomatic cyst carriers because statistics reveals up to 10% of individuals with asymptomatic *E. histolytica* colonisation developed into amoebic colitis or extraintestinal disease (Gathiram and Jackson, 1987; Haque *et al.*, 2001).

The clinical manifestation of amoebic colitis depends on the depth of the invasion which may vary from local mucosal erosion to mucosal ulceration. In intestinal amoebiasis cases, some patients showed intermittent diarrhoea which alternates with constipation. Fever is uncommon for *E. histolytica* infection, in which less than 40% were reported among amoebic patients (Adams and MacLeod, 1977). At the benign phase of intestinal amoebiasis, an individual may experience only mild to severe diarrhoea. Diarrhoea may be replaced by dysenteric stools when the lesion of mucosal increases. Occasionally, local invasion of trophozoites could induce a proliferative granulomatous response at the ulcerative site and turn it into a pseudo-tumour which also known as amoeboma (Sodeman, 1996).

1.3 Nonpathogenic *Entamoeba* species

The nonpathogenic *Entamoeba* species that were found in human body included *E. dispar*, *E. moshkovskii*, *Entamoeba coli*, *Entamoeba gingivalis*, *Entamoeba polecki*, *Entamoeba hartmanni* and *Entamoeba chattoni* (Zlobl, 2001). Table 1.3 and Figure 1.3 show the summarised descriptions of these common amoebas.

Table 1.3 Morphologic features and pathogenicity of intestinal amoeba

Characteristics	***E. histolytica, E. dispar* and *E. moshkovskii***	***E. hartmanni***	***E. coli***	***E. polecki***	***E. nana***
Trophozoites (size, nucleus, and movement)	15 to 20 μm; one nucleus; actively motile cytoplasmic protrusions, quickly finger shaped pseudopodium	8 to 10 μm; one nucleus; nonsuccessive	20 to 25 μm; one nucleus; slow movement, short and blunt pseudopodium	15 to 20 μm; one nucleus; motility resembles *E. coli*	7 to 9 μm; one nucleus, blunt and hyaline pseudopodium, slow movements
Cysts (size, nucleus)	12 to 15 μm; mature cyst has four nuclei; immature cyst has one or two nuclei	6 to 8 μm; mature cyst has four nuclei; immature cyst has one or two nuclei; two nucleated cysts very common	15 to 25 μm; mature cyst has eight nuclei, rarely 16 or more nuclei	10 to 15 μm; one nucleus, very rarely binucleated or quadrinucleated	6 to 8 μm; one 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, four 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

Adapted from (Tanyuksel and Petri, 2003)

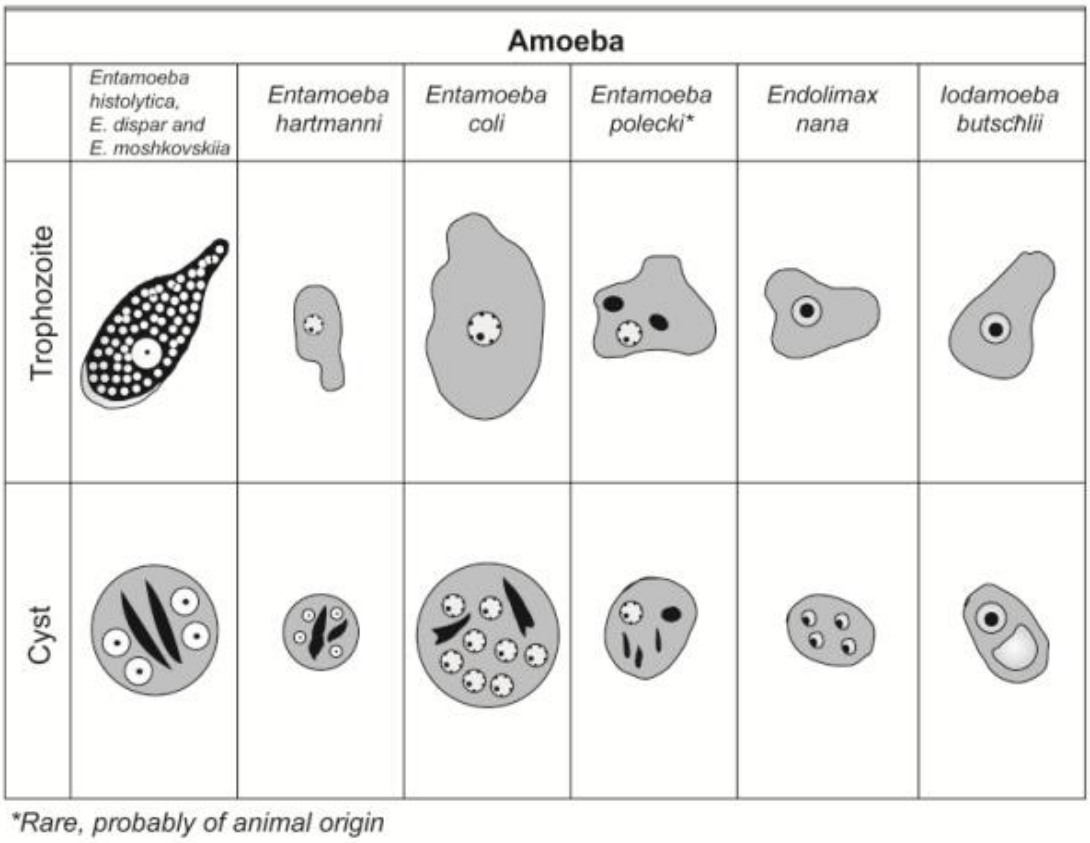

Figure 1.3 Amoebas found in stool specimens of humans

Adapted from (Baron, 1996)

Among these *Entamoeba* species, only *E. dispar* and *E. moshkovskii* gained the attention from clinicians and researchers as both amoebas are morphologically indistinguishable from *E. histolytica* by microscopy. Both can only be differentiated at the molecular level as they are genetically distinct.

E. dispar was first described by Brumpt in 1925 as a *E. histolytica*-like nonpathogenic strain (Jackson, 1998; Ackers, 2002). However, Diamond and Clark described it as a new species although it was closely related to *E. histolytica* (Diamond and Clark, 1993). Later, this nonpathogenic *E. histolytica* was renamed as *E. dispar* by Diamond and Clark in 1993 (Diamond and Clark, 1993; Stauffer and Ravdin, 2003; Tanyuksel and Petri, 2003). In 1997, *E. dispar* was officially classified as a nonpathogenic *Entamoeba* species by World Health Organization (WHO, 1997). Despite being claimed as a nonpathogenic *Entamoeba* species, some patients infected with *E. dispar* showed intestinal amoebiasis symptom (Jetter *et al.*, 1997). It was able to produce variable focal intestinal lesions in animals and can rupture monolayer of epithelial cell *in vitro* (Chadee *et al.*, 1985; Vohra *et al.*, 1989; Espinosa Cantellano *et al.*, 1997; Espinosa-Cantellano *et al.*, 1998). Although McMillan *et al.* (1984) had reported *E. dispar* as a human pathogen, there was no further verification due to the absence of large case-controlled studies on the this species.

A couple of pathogenicity studies of *E. dispar* were demonstrated using animal model, in which similar hepatic and intestinal lesions were observed to those caused by *E. histolytica* (Costa *et al.*, 2000; Gomes *et al.*, 2000). To make it worse, *E. dispar* strains isolated from asymptomatic patients were reported to have successfully induced ALA experimentally in hamsters (Dolabella *et al.*, 2012; Guzman-Silva *et al.*, 2013). However, Oliveira *et al.* (2015) suggested that more further studies should be conducted on the association between pathogenic enterobacteria with *E. dispar* in

favouring the expression of amoebic virulence factors responsible for the formation of epithelial lesions which favour the invasion and adhesion of *E. dispar*. This is because, pathogenic enterobacteria were found to be able to increase the virulence of *E. histolytica* and the same thing might happen to *E. dispar* (Galvan-Moroyoqui *et al.*, 2008).

E. moshkovskii was first described by Tshalaia in 1941 from Moscow sewage. Since then the amoeba was reported in many other different countries (Scaglia *et al.*, 1983; Clark and Diamond, 1991). *E. moshkovskii* was initially thought to be a free-living environmental strain until an *E. histolytica*-like strain was found and isolated from a resident who stayed in Laredo, Texas (Dreyer, 1961). It was named as *E. histolytica* Laredo strain due to its osmotolerant and resistant to emetine capacity that distinguished it from *E. histolytica* and *E. dispar* (Clark and Diamond, 1991; Clark and Diamond, 1997). Subsequently, this strain was confirmed and recognised as *E. moshkovskii* through molecular studies (Clark and Diamond, 1991). Some recent studies have reported the recovery of *E. moshkovskii* from human faeces based on microscopy, which makes the reports confusing as it cannot be distinguished from *E. histolytica* (Ali *et al.*, 2003; Parija and Khairnar, 2005; Fotedar *et al.*, 2007b; Tanyuksel *et al.*, 2007). Although a study from India shows *E. moshkovskii* as a sole potential enteropathogen in patients, however further studies should be performed to investigate its pathogenic potential in human (Parija and Khairnar, 2005).

1.4 Laboratory diagnosis of amoebiasis

Traditionally, the diagnosis of amoebiasis is based on microscopic examination of biological samples. Detection of the pathogen then was shifted to molecular biology-based diagnostic tests as the technology advances. Various protein detection

assays were developed, which include enzyme-linked immunosorbent assay (ELISA), indirect haemagglutination assay (IHA) and latex agglutination. The introduction of DNA detection-based assays has helped to overcome the weakness of microscopy to enable the detection and differentiation of *E. histolytica* from other nonpathogenic *Entamoeba* species (Petri and Singh, 1999; Fotedar *et al.*, 2007a).

1.4.1 Microscopy

Microscopic examination of stool samples is the most common routine diagnostic method used in many parasitology laboratories for diagnosis of both intestinal and extraintestinal amoebiasis. Stool examination should be done within 4 h before the trophozoites die of environmental stress (Tan *et al.*, 2010). The routine microscopic stool observation method involves three common steps, which are wet preparation, concentration, and permanently stained smears. The cysts and trophozoites observation using direct wet mount is simple and easy to perform despite its low sensitivity of only up to 60% (Fotedar *et al.*, 2007a). This method can be further verified with identification of trophozoites containing red blood cells for patients with dysentery. However, erythrophagocyted trophozoites will only be observed in stool samples of patients presented with acute dysentery (Huston *et al.*, 1999). Besides, stool sample with *E. dispar* containing red blood cells were also reported (Haque *et al.*, 1995). Despite the well-established modified iron haematoxylin permanent stains and Wheatley trichrome staining techniques (Fotedar *et al.*, 2007a), eosin Y staining method was reported to be more efficient and easier to perform (Tan *et al.*, 2010). Eosin Y could rapidly stain the trophozoites with light red colour, while the central karyosome and chromatin materials were stained distinctly dark. Experienced and skillful personnel has always been important criteria for microscopic examination and

interpretation of results (Barrett-Connor, 1971; Mukhopadhyay *et al.*, 2000). Presence of nonpathogenic *Entamoeba* species further increase the application doubt for identification of *E. histolytica* using microscopic examination (Haque *et al.*, 1995; WHO, 1997; Tanyuksel and Petri, 2003; Haque and Petri, 2006; Liang *et al.*, 2009). Therefore, a microscopic examination result should be verified by molecular assay (Fotedar *et al.*, 2007a).

1.4.2 Biochemical methods

Biochemical method uses artificial enriched media such as TYI-S-33, Locke-egg medium and TYSGM-9 to axenically culture the isolated trophozoites from patient, followed by isoenzyme analysis. Axenic culture of *E. histolytica* involves growing of the parasites in a medium without the presence of any microorganism except the intended protozoa. This method could distinguish the pathogenic *E. histolytica* from other nonpathogenic *Entamoeba* species (Sargeaunt *et al.*, 1978; Fotedar *et al.*, 2007a). Isoenzyme analysis differentiates *Entamoeba* spp. based on zymodeme. Zymodeme is defined as a group of ameba strains that share the same electrophoretic pattern and mobilities for several enzymes namely phosphoglucomutase, hexokinase, malic enzyme and glucose-6-phosphate isomerase (Sargeaunt *et al.*, 1987; Razmjou *et al.*, 2006). Twenty-four different zymodemes have been described, of which 21 are from human isolates (12 of *E. dispar* and 9 of *E. histolytica*) and another three are from experimentally cultured amoeba strains.

Although this method can differentiate *E. histolytica* from *E. dispar*, axenic culture of *E. histolytica* is not feasible in a clinical diagnostic laboratory due to its time-consuming process (Clark and Diamond, 2002). Moreover, culture technique is not only labour-intensive, it may not always be successful (Ackers, 2002; Clark and

Diamond, 2002; Haque and Petri, 2006). The selection bias on cultivated amoeba may generate false negative result due to outgrown of non-*Entamoeba* species during culture (Gonzalez-Ruiz *et al.*, 1994; Haque *et al.*, 1995). Furthermore, zymodeme analysis is low in analytical sensitivity or high in limit of detection as it requires large number of cells for the enzyme analysis. Therefore, isoenzyme analysis is no longer suitable for diagnosis of amoebiasis.

1.4.3 Antibody detection

Serological method is useful for diagnosis of amoebiasis in non-endemic countries where background amoebic antibodies are low. However, application of serological diagnosis is a challenge in amoebiasis endemic areas. This is because *Entamoeba* antibody detection is unable to differentiate recent infection from past infection in endemic areas (Caballero-Salcedo *et al.*, 1994; Pillai *et al.*, 1999). However, serological method was reported to be 100% sensitive based on screening of anti-amoebic antibodies in serum samples at China (Zengzhu *et al.*, 1999). There are various antibody detection tests for amoebiasis such as counterimmuno-electrophoresis, ELISA, indirect immunofluorescence assay, IHA, amoebic gel diffusion, indirect fluorescent assay, complement fixation and latex agglutination (Fotedar *et al.*, 2007a). Among them, ELISA is the most commonly used format because it is highly sensitive for antibody detection and can be fully automated. It was reported to be useful for diagnosis of extraintestinal infections when amoeba cannot be detected in faeces (Rosenblatt *et al.*, 1995). In addition, it can be easily performed in a clinical laboratory (Fotedar *et al.*, 2007a).

1.4.4 Antigen detection

Antigen detection method could differentiate *E. histolytica* and *E. dispar* based on presence of lectin (Haque *et al.*, 1997; Haque *et al.*, 1998). TechLab *E. histolytica* II (TechLab, Blacksburg, VA) is a commercial antigen detection test for diagnosis of amoebiasis. This kit was reported to have high specificity of between 93 to 100% and sensitivity of up to 100% (Fotedar *et al.*, 2007a). Besides its application on stool, this kit was reported to detect circulatory lectin antigen in serum, saliva and aspirated liver pus samples (Haque and Petri, 2006; Fotedar *et al.*, 2007a). However, study conducted by Zeehaida *et al.* (2008) revealed that TechLab *Entamoeba histolytica* II kit only worked effectively on ALA patients who have yet to receive treatment prior to specimen collection. Another study demonstrated that this kit recorded a sensitivity of only up to 14.3% for *E. histolytica* detection compared to zymodeme and culture identification (Gatti *et al.*, 2002). In addition, false positive results of this test were also being suspected (Furrows *et al.*, 2004; Visser *et al.*, 2006). Hence, the specificity and sensitivity of antigen detection using the TechLab kits should be re-evaluated (Gatti *et al.*, 2002; Gonin and Trudel, 2003).

1.4.5 DNA-based detection

DNA-based detection method is usually accompanied by DNA amplification process such as PCR. This method is being endorsed by WHO for detection of *E. histolytica* and has been widely studied and utilised in developed and some developing countries (Zaki *et al.*, 2002; Calderaro *et al.*, 2006; Hamzah *et al.*, 2006; Haque and Petri, 2006). It can detect *E. histolytica* in variety of clinical specimens, and even differentiate *E. histolytica* from other morphologically distinguishable nonpathogenic *Entamoeba* species (Tanyuksel and Petri, 2003; Fotedar *et al.*, 2007a; Parija *et al.*,

2014). Many genes have been described for detection of *E. histolytica* such as 30-kDa surface antigen gene, small-subunit ribosomal RNA genes, M17 genes, and some extrachromosomal circular DNA genes. Among those genes, small-subunit ribosomal RNA is the most widely used gene sequence due to its multi-copy and extrachromosomal features (Bhattacharya *et al.*, 1989; Fotedar *et al.*, 2007a).

Initially, PCR approach was merely used to specifically identify the pathogenic *E. histolytica*. However, conventional single target PCR was found to be time-consuming and labour-intensive since separate PCR runs are required to identify different species of *Entamoeba*. Since then, PCR-based detection method has been transformed into multiple advanced formats such as multiplex PCR, nested PCR and real-time PCR. Multiplex PCR is a platform that offered simultaneous detection of two or more targets in a single reaction by using more than a pair of primers. A single round PCR assay developed by Hamzah *et al.* (2006) was a good example where this assay was reported to be able to detect *E. histolytica*, *E. dispar* and *E. moshkovskii* simultaneously. Simultaneous multiple target amplification is benefiting as co-infection between *E. histolytica* and *E. dispar* did occur. For example, a study in Cuba reported that with 24.5% of those screened had mixed *E. histolytica* and *E. dispar* infection (Nunez *et al.*, 2001). Another study by Haque *et al.* (1998) found 18% of mixed infection cases in stool samples collected from symptomatic patients at Dhaka, Bangladesh.

Real-time PCR is a highly sensitive but sophisticated machine-dependent amplification technique. Unlike conventional PCR, this technique does not require agarose gel electrophoresis to analyse its product. Instead, it uses sophisticated computerised machine to amplify and simultaneously quantify their targeted DNA molecule. Real-time PCR amplification was reported to be sensitive and specific for

diagnosis of amoebiasis. In addition, it offers direct sample analysis while amplification is still ongoing hence eliminated the need for post-PCR analysis and reduces the total assay time (Fotedar *et al.*, 2007a). Various real-time PCR tests have been published for detection of *E. histolytica* (Roy *et al.*, 2005; Haque *et al.*, 2007) with some even possess the ability to differentiate among *Entamoeba* species (Lau *et al.*, 2013; Gomes Tdos *et al.*, 2014).

Non-specific or cross-amplification has always been the concern for detection of *E. histolytica* in stool sample. This is because DNA from other organisms may be isolated from stool and get detected if the primers involved in amplification are not specific. Hence, nested PCR offered a better specificity platform by having two sets of primers to amplify a gene target in two successive runs. Khairnar and Parija (2007) have developed a novel nested multiplex PCR assay for differential detection of *E. histolytica*, *E. moshkovskii* and *E. dispar* in stool samples. This combination of multiplex PCR and nested PCR assay offers multiple targets detection with higher specificity in a single reaction. To further reduce the multiple pipetting steps during PCR preparation and to solve the time-consuming two successive runs of PCR, a ready-to-use single reaction cycle nested multiplex PCR assay was developed by Foo *et al.* (2012).

Unlike other detection platform, DNA-based detection assays could be applied on stool samples with or without preservative (Tanyuksel and Petri, 2003; Fotedar *et al.*, 2007a). PCR had also been reported for detection of *E. histolytica* in aspirated pus/abscess, urine and saliva (Ahmad *et al.*, 2007; Parija and Khairnar, 2007; Othman *et al.*, 2010). One of the disadvantages of using PCR method to detect *E. histolytica* in faecal samples is due to the presence of amplification inhibitors. The amplification inhibitors in form of molecules complexes such as bile salts, bilirubins, heme and even

carbohydrates could be co-extracted along with the pathogen DNA (Holland *et al.*, 2000). A new amplification approach which uses single temperature to amplify target gene has further simplified the process of DNA amplification process. This loop-mediated isothermal amplification (LAMP) which only require a single temperature to complete the strand displacement, annealing and extension processes made it optionally eliminates the need of thermal cyclers (Notomi *et al.*, 2000; Fotedar *et al.*, 2007a). This method uses DNA polymerase that perform displacement activities without denaturising the double-stranded DNA requires only 30 to 60 min to amplify a target gene (Notomi *et al.*, 2000). Liang *et al.* (2009) has reported that a LAMP assay was as sensitive and specific as the conventional nested PCR for detection of *E. histolytica* in clinical stool specimens. Another LAMP assay for detection of *E. histolytica* was then developed by Rivera and Ong (2013) and the authors claimed that the assay could detect as low as five parasites per reaction. A LAMP assay applicability assessment was conducted by Singh *et al.* (2013) on specimens with clinical suspicion of ALA. The comparison assessment done with conventional PCR showed LAMP have better detection sensitivity. Hence, LAMP amplification platform is a potentially reliable tool for diagnosis of amoebiasis.

1.5 Point-of-care diagnostic test

The emergence of point-of-care or ready-to-use diagnostic platform offers spontaneous result analysis with minimal machine dependency. This diagnostic platform was deemed relevant for better disease control in the developing countries as it is Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free and Deliverable (ASSURED) (Wu and Zaman, 2012). The initial priorities for development of 'ASSURED' diagnostics focused on sexually transmitted diseases

such as syphilis, gonorrhoea and chlamydia (Kettler *et al.*, 2004). The point-of-care diagnostic platform could also optionally avoid the need of huge and expensive equipment such as ELISA reader, gel electrophoresis system and thermal cycler. Thus, ASSURED complied tests are the preferred choice for diagnosis of infection disease in developing country with low resources setting.

A diagnostic assay made of a molecular amplification method combined with a lateral flow device is one of the current practicable diagnostic platform. This is because of the practicality of result analysis using lateral flow strips in low resource setting including highly portable, simple to use, cheap to produce and produce rapid visual result characteristics. Reported point-of-care diagnostic assays such as dry-reagent lateral flow for the detection of *Vibrio cholerae* with PCR (Chua *et al.*, 2011a) and LATE-PCR with dry-reagent nucleic acids-based lateral flow assay (Ang *et al.*, 2015) have shown good potential as they were highly sensitive and specific. However, the PCR amplification incorporated in these assays made them dependent on thermal cycler. Various studies were conducted to obviate the use of thermal cycler. A combination of LAMP amplification with lateral flow technology was reported to have work as efficiently as PCR-lateral flow assays (Surasilp *et al.*, 2011). Since then, many assays were reported for various targets based on this LAMP-lateral flow combination (Rigano *et al.*, 2014; Yongkiettrakul *et al.*, 2014; Thongkao *et al.*, 2015; Najian *et al.*, 2016). Incorporation of LAMP amplification in replacing PCR not only eliminated the needs of sophisticated machine (thermal cycler), it also cuts short the amplification duration as LAMP amplification performance is much higher than PCR (Notomi *et al.*, 2000).

1.5.1 Loop-mediated isothermal amplification

Generally, DNA-based isothermal amplification or single temperature amplification is divided into four different categories, namely LAMP, strand displacement amplification (SDA), helicase-dependent amplification (HDA) and nicking enzyme amplification reaction (NEAR). The differences among these isothermal platforms were involvement of different enzymes that assisted strand-displacement and primers design. Among those, LAMP is the most frequently reported isothermal amplification platform for detection of pathogens (Yan *et al.*, 2014). LAMP uses high strand displacement activity DNA polymerase, an *in silico* designed homologue of *Bacillus stearothermophilus* to catalyse the auto-cycling stand-displacement activity under a single temperature ranging 55 to 65°C (Notomi *et al.*, 2000). This amplification provides high target specificity because it uses four to six primers that recognise six to eight distinct regions of target DNA (Notomi *et al.*, 2000; Nagamine *et al.*, 2002). There are three different primer pairs involved in LAMP, which are inner primers (form with F1c-F2 or B1c-B2 target regions), outer primers (form with F3 or B3 target region) and loop primers as illustrated in Figure 1.4.

The amplification process starts when the F2 region of the inner primer hybridises to the target and the DNA sequence extended by *Bst* DNA polymerase. Displacement process takes place when the outer primer binds to F3 complementary (F3c) region of the same target and extended the F3 DNA sequence to further displace the newly synthesised strand. The first displaced strand will then form a stem-loop structure at the 5’ end due to the hybridisation of F1c and F1 region. A new strand with stem-loop structure at both ends is generated when the backward inner primer and outer primer repeated the displace-amplification process at the 3’ end of the previous strand. This process produces a dumbbell-like structured DNA which later

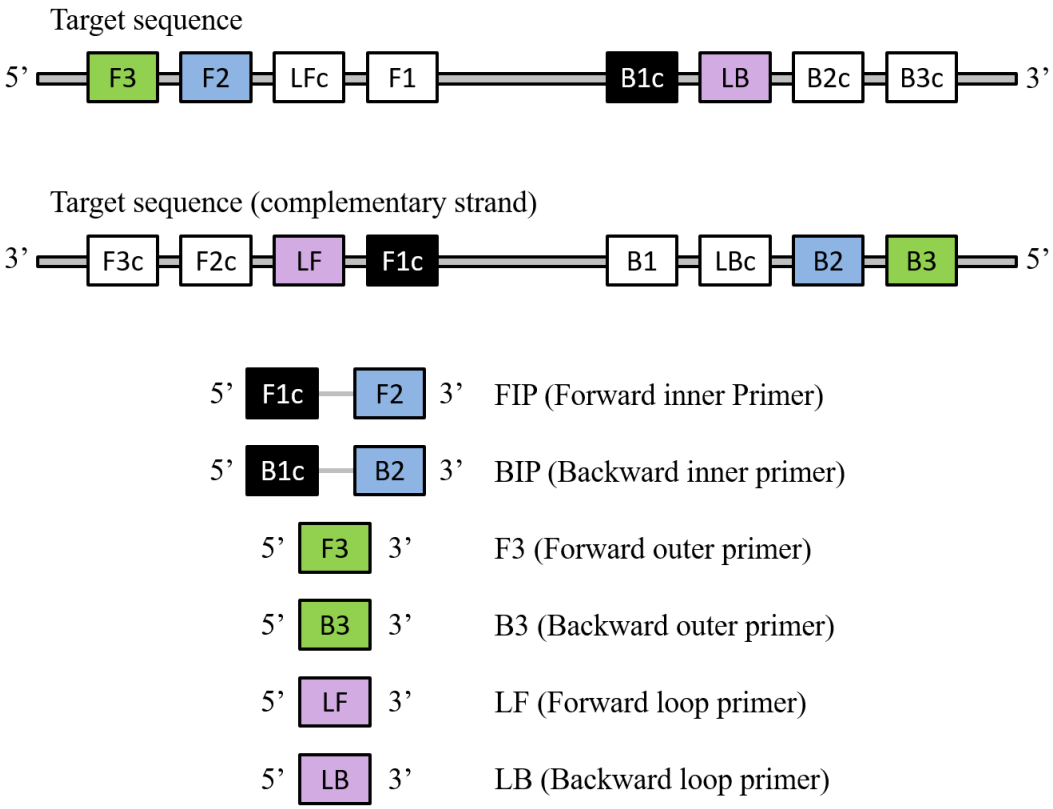

Figure 1.4 Primers for loop-mediated isothermal amplification

involved in series of amplification cycle that beyond exponential (Notomi *et al.*, 2000). To further improve the amplification performance, a pair of loop primers were suggested to be incorporated. This set of primer will join into the amplification process by binding at the stem-loop of the dumbbell-like structure extended their sequence to form a brand new double-stranded structure. Inclusion of loop primers not only improve the amplification, additional primers that recognises specific sequence would improve its specificity of the amplification (Nagamine *et al.*, 2002). Even when large amounts of non-target DNA are present, LAMP was still able to amplify up to 10^9 copies of the target within one hour (Notomi *et al.*, 2000). Thus, LAMP has been widely applied to rapidly detect pathogens, including *E. histolytica* (Liang *et al.*, 2009; Rivera and Ong, 2013; Singh *et al.*, 2013). Although these assays were reported to be as sensitive as PCR platform for detection of *E. histolytica*, they are still dependent on sophisticated equipment such as electrophoresis system, turbidimeter and UV light-related analyser.

1.5.2 Lateral Flow

The lateral flow-based nucleic acid detection platform can be classified into competitive and non-competitive approaches. Non-competitive platform can be further classified based on the type of immobilised capture reagent on the lateral flow strip. A lateral flow system with immobilised hapten or antibody on the reaction pad is named nucleic acid lateral flow immunoassay (NALFIA) whereas lateral flow system with immobilised oligonucleotides on the reaction pad is referred as nucleic acid lateral flow (NALF) assay. NALFIA can be further diverged into hybridisation and non-hybridisation categories. NALFIA that detect double-labelled amplicon with probe hybrids is classified as hybridisation-based while assays that detects double-labelled