AN INVESTIGATION OF DIFFERENTIALLY ABUNDANT MEMBRANE PROTEINS IN VIRULENT VERSUS AVIRULENT VARIANTS OF *Entamoeba histolytica* HM-1:IMSS

by

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LIST OF ABBREVIATIONS

2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
ACN	Acetonitrile
ADH	Alcohol dehydrogenase
ALA	Amoebic liver abscess
APS	Ammonium persulfate
BSA	Bovine Serum Albumin
CHCA	α-Cyano-4-hydroxycinnamic acid
CID	Collision induced dissociation
CBB	Coomassie Brilliant Blue
CP	Cysteine proteinase
cRAP	Common Repository of Adventitious Proteins
Da	Dalton
DIGE	Differential gel electrophoresis
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization

FDR	False discovery rate
Gal	Galactose
GalNAc	N-acetyl-D-galactosamine
GPI	Glucose-6-phosphate isomerase
HCD	Higher-energy collisional dissociation
HCl	Hydrochloric acid
IAA	Iodoacetamide
IHA	Indirect haemagglutination assay
iTRAQ	Isobaric tags for relative and absolute quantitation
KCl	Potassium chloride
KH_2PO_4	Potassium phosphate
LC	Liquid chromatography
m/z	Mass-to-charge ratio
MALDI	Matrix-assisted laser desorption/ionization
MMTS	Methyl methanethiosulfonate
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NaN_3	Sodium azide
PANTHER	Protein ANalysis Through Evolutionary Relationships

PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PTM	Post-translational modification
RCDC	Reducing agent compatible and detergent compatible
RP	Reverse phase
S/N	Signal-to-noise ratio
SDS	Sodium dodecyl sulfate
SILAC	Stable isotope labelling with amino acids in cell culture
spp	Species (plural)
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
TEAB	Triethylammonium bicarbonate
TEMED	Tetramethylethylenediamine
TFA	Trifluoroacetic acid
TM	Transmembrane
TOF	Time of flight
TOF/TOF	Tandem time of flight
TPCK	6- (1-tosylamido-2-phenyl) ethyl chloromethyl ketone
TYI-S-33	Trypticase-Yeast Extract-Iron-Serum

KAJIAN TENTANG PERBEZAAN KELIMPAHAN PROTEIN MEMBRAN DALAM VARIAN VIRULEN MELAWAN AVIRULEN *Entamoeba histolytica* HM-1:IMSS

ABSTRAK

Entamoeba histolytica adalah parasit protozoa yang menyebabkan amebiasis dan meningkatkan risiko kesihatan secara signifikan kepada populasi manusia di kawasan endemik. Mekanisme molekul yang terlibat dalam pengawalaturan terhadap patogenesis parasit ini belum dipercirikan dengan baik. Oleh yang demikian, kajian ini bertujuan untuk menjalankan identifikasi dan kuantifikasi terhadap protein membran dengan kelimpahan berbeza dengan membandingkan protein membran *E. histolytica* HM-1:IMSS yang terdiri daripada varian virulen dan avirulen. Kajian interaksi yang berpotensi antara protein membran yang berlainan kelimpahan turut dilakukan. Kajian ini menggunakan kaedah proteomik kuantitatif dengan menggunakan pelabelan Isobaric Tags for Relative and Absolute Quantitation (iTRAQ), dengan kombinasi dua instrumen spektrometri jisim iaitu nanoLC-MALDI-MS/MS dan nanoLC-ESI-MS/MS. Secara amnya, 37 protein membran menunjukkan perbezaan yang tinggi, di mana 19 dan 18 protein membran masing-masing daripada *E. histolytica* varian virulen menunjukkan peningkatan dan penurunan kelimpahan berbanding varian avirulen. Contoh protein membran dengan kelimpahan berbeza termasuk Rho family GTPase, calreticulin, protein kerintangan pelbagai ubat dan protein hipotetikal. Analisa gen ontologi oleh PANTHER mendedahkan secara umumnya protein membran dengan kelimpahan berbeza terlibat dalam aktiviti katalitik (29.72%) dan proses metabolik (32.43%). Peranan putatif bagi protein-protein tersebut ditentukan berdasarkan kevirulenan *E. histolytica*. Kesimpulannya, kajian ini memberi profil protein membran

secara mendalam antara *E. histolytica* HM1:IMSS varian virulen dan avirulen. Tambahan pula, pengenalpastian protein membran dengan kelimpahan berbeza dapat membantu dalam penjelasan faktor kevirulenan *E. histolytica*.

AN INVESTIGATION OF DIFFERENTIALLY ABUNDANT MEMBRANE PROTEINS IN VIRULENT VERSUS AVIRULENT VARIANTS OF *Entamoeba histolytica* HM-1:IMSS

ABSTRACT

Entamoeba histolytica is a protozoan parasite that causes amoebiasis and poses a significant health risk for human populations in the endemic areas. The molecular mechanisms involved in the pathogenesis regulation of the parasite are not well characterized. Hence, this study was aimed at identifying and quantifying the differentially abundant membrane proteins by comparing the membrane proteins of virulent and avirulent variants of *E. histolytica* HM-1:IMSS. Additionally, investigation on the potential interactions among the differentially abundant membrane proteins was performed. Quantitative proteomics approach using Isobaric Tags for Relative and Absolute Quantitation (iTRAQ) labelling was employed in combination with two mass spectrometry instruments *i.e.* nanoLC-MALDI-MS/MS and nanoLC-ESI-MS/MS to identify the proteins. Overall, 37 membrane proteins were found to be differentially abundant, where 19 and 18 membrane proteins of the virulent variant of *E. histolytica* increased and decreased in abundance, respectively when compared to the avirulent variant. Examples of membrane proteins that were differentially abundant include Rho family GTPase, calreticulin, multidrug resistance proteins and hypothetical proteins. Gene ontology analysis by PANTHER revealed that the differentially abundant membrane proteins were predominantly involved in the catalytic activities (29.72%) and metabolic processes (32.43%). The putative roles of the differentially abundant membrane proteins involved primarily in the catalytic activities and metabolic processes were determined in relation to the virulence of *E.*

histolytica. In conclusion, this study provided an in-depth membrane protein profiling comparison between the virulent and avirulent variants of *E. histolytica* HM-1:IMSS. Moreover, identification of the differentially abundant membrane proteins could lead the elucidation of *E. histolytica* virulence factors.

CHAPTER 1

INTRODUCTION

1.1 Background

Entamoeba histolytica is an enteric protozoan parasite that causes invasive infection in natural hosts including human and several higher non-human primates (Stanley, 2003; Verweij et al., 2003; Schuster & Visvesvara, 2004). Amoebiasis is a disease caused by *E. histolytica* (Pritt & Clark, 2008), which poses a health risk affecting 40-50 million of individuals in the world population, and causes approximately 100,000 of death per annum globally (Walsh et al., 1986; WHO, 1997). Globally, amoebiasis is the second leading deadliest diseases due to the parasitic infection. The highest morbidity and mortality cases were recorded in tropical and subtropical countries, such as Vietnam, Mexico, and India (Widmer & Nettleman, 1991), where personal hygiene and sanitation are often neglected.

The epidemiology of amoebiasis in tropical regions mainly affects the general population. Meanwhile, in developed countries, the incidence of amoebiasis tends to be prevalent among elderly patients, travelers to and from endemic regions, homosexuals male and immunosuppressed or institutionalized individuals (Reed, 1992; Petri, 1999; Hung et al., 2008; Mitchell & Hughes, 2018). The worldwide assessment on the prevalence of *E. histolytica* infection revealed that 90% of the infected individuals remain asymptomatic, while approximately 10% of the infected individuals developed into the clinically apparent diseases (Jackson et al., 1985; Haque & Petri, 1999). In Malaysia, the prevalence of parasites infections among different aborigine populations has been studied. A study on an intestinal parasite prevalence was carried out among the aborigine communities in Pos Piah, Perak showed that 11.5% of the population was infected with *E. histolytica/E. dispar* (Noor Hayati et al., 1998).

In Gombak, the parasitic infection incidence was assessed among school children aged from 6 to 13. From the result, *E. histolytica* (9.9%) was the fourth most common parasite found in the children after *Trichuris* (47.1%), *Giardia intestinalis* (14.7%) and *Entamoeba coli* (11.4%) (Rajeswary et al., 1994). In Pahang, the prevalence of *E. histolytica* among children and adults was 18.5% (Yusuf et al., 2007). In addition, 21% of *E. histolytica* prevalence was obtained from villagers of Sabah, East Malaysia (Aza et al., 2003). Generally, the prevalence of parasites including *E. histolytica* and *E. dispar* in Malaysia was higher in the rural community when compared to the urban community. For example, Jamaiah & Rohela (2005) reported that the prevalence of intestinal parasites among the community in Kuala Lumpur was only 0.4% (Jamaiah & Rohela, 2005).

E. histolytica has been deliberated as a potential pathogenic protozoon parasite that can cause severe damage to the intestine like intestinal colitis and dysentery, as well as other extra-intestinal organs, predominantly the liver such as amoebic liver abscess (ALA) (Ximénez et al., 2010). The ALA is the most common form of the extra-intestinal amoebiasis (Rao et al., 2009). Another manifestation of amoebiasis is the Pleuropulmonary amoebiasis, which results from the contiguous spread from a liver abscess rupturing through the right hemidiaphragm (Loulergue et al., 2009). It is the second most common extra-intestinal expression after ALA (Faye et al., 2011).

Poor personal hygiene practice and sanitation increase the risk of *E. histolytica* infection, as the major mode of transmission is by the consumption of *E. histolytica* cyst-contaminated food or water. Cysts can also be detected in faecally-contaminated soil, fertilizer or from the contaminated hands of food handlers. Another transmission route also possible, which is via faecal-oral route. This commonly occurs among

individuals that practice anal sexual or direct rectal inoculation by the colonic irrigation tools.

The cyst will stay viable in the host environment for weeks to months, and when an excystation occurs, the invasive form trophozoites will be released in the large intestine. These trophozoites live in the terminal ileum or colon and can penetrate through the colonic mucosal barrier causing mucosal defect, resulting in the formation of the flask-shape ulcer with necrosis in the submucosa, resembling inflammatory bowel disease (Stanley, 2003).

To date, the mechanism of *E. histolytica* that causes amoebiasis is still unclear. Therefore, several comparative proteomics studies on *E. histolytica* have been performed to elucidate the virulence factors (Davis et al., 2006; Davis et al., 2009; Valdés et al., 2014; Perdomo et al., 2015). These studies utilized the two-dimensional gel-based (DIGE) and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and had revealed several significant virulence factors of *E. histolytica* including peroxiredoxin and alcohol dehydrogenase 3. However, the gel-based method has substantial limitations for instance insufficient sample load capacity and poor separation of hydrophobic, acidic and alkaline proteins (Bunai & Yamane, 2005). Hence, some of the important proteins might lose and affect the analysis. Therefore, a more advanced proteomic analysis approach such as quantitative proteomics have been introduced to overcome these challenges.

Quantitative proteomics using the non-gel based approach has emerged as a robust and powerful technology that has been utilized in many studies, particularly for biological marker discovery and targeted proteomic studies. Commonly, the quantitative proteomics technology has been applied using with or without specific

tagging or labelling. For instance, the metabolic labelling technique was utilized to examine the variations in the *Plasmodium falciparum* proteome during the cell cycle (Nirmalan et al., 2004).

On the other hand, the iTRAQ reagents that were used in peptide tagging were designed to allow more confident protein identification and quantification, thus enhancing the peptides coverage of the protein sample (Zieske, 2006).

1.2 Statement of Problem and Rationale of Study

Membrane proteins, including integral proteins and membrane-associated proteins, play significant roles in many biological functions of *E. histolytica*. The membrane proteins contain either transmembrane domains and/or signal peptides that are responsible for membranous trafficking as well as for vesicular trafficking machinery (Krogh et al., 2001). Currently, the mechanisms underlying pathogenicity of *E. histolytica* have not been fully understood. Previous studies have characterized a number of amoebic virulence determinants. The virulent determinants include a member of membrane proteins that play an essential role in the pathogenicity of *E. histolytica*, such as galactose/N-acetyl galactosamine inhibitable lectin (Gal/GalNAc lectin), cysteine proteases and phagosome-associated proteins. Gal/GalNAc lectin was reported to be substantial for adherence of trophozoites to host cells, cytolysis, parasite invasion, and phagocytosis (Boettner et al., 2002; Mann et al., 2002). Cysteine proteases have been involved in cytopathic activities like destructing host cells and tissues. They also stimulate the intestinal inflammation and degradation of the host extracellular matrix (DeMeester et al., 1990; Mortimer et al., 2010). Meanwhile, phagosome-associated proteins such as EhRac A, EhPAK (p21-activated serine/threonine protein kinase), and actin have been reported to be involved in endocytosis and disease pathogenesis (Laughlin & Temesvari, 2005).

To date, there are still many putative and hypothetical membrane proteins that are involved in the virulence mechanisms with unknown function and have not been identified and characterized. Therefore, to increase the understanding of the disease pathogenesis, these proteins need to be identified and further investigated so that the factors contributing to the outcome of the infection and development of the invasive amoebiasis would be better understood.

To investigate the differentially abundant proteins in virulent versus avirulent variants, the application of iTRAQ labelling approach in this study is expected to improve the overall proteome coverage which is important for protein profiling studies and uncovering the potential virulence factors from the differentially abundant membrane proteins.

1.3 Objectives of the Study

The objectives of this study are as follows:

1. To identify the differentially abundant membrane proteins from the virulent *versus* avirulent variants of *E. histolytica* HM-1:IMSS by using iTRAQ quantitative proteomics, in combination with nanoLC-MALDI-TOF/TOF and nanoLC-ESI-MS/MS.

2. To classify the differentially abundant membrane proteins using PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System annotations based on the biological process and molecular function.

3. To analyse the protein-protein interactions among the differentially abundant membrane proteins using Search Tool for the Retrieval of Interacting Genes/Proteins (STRING).

1.4 Workflow

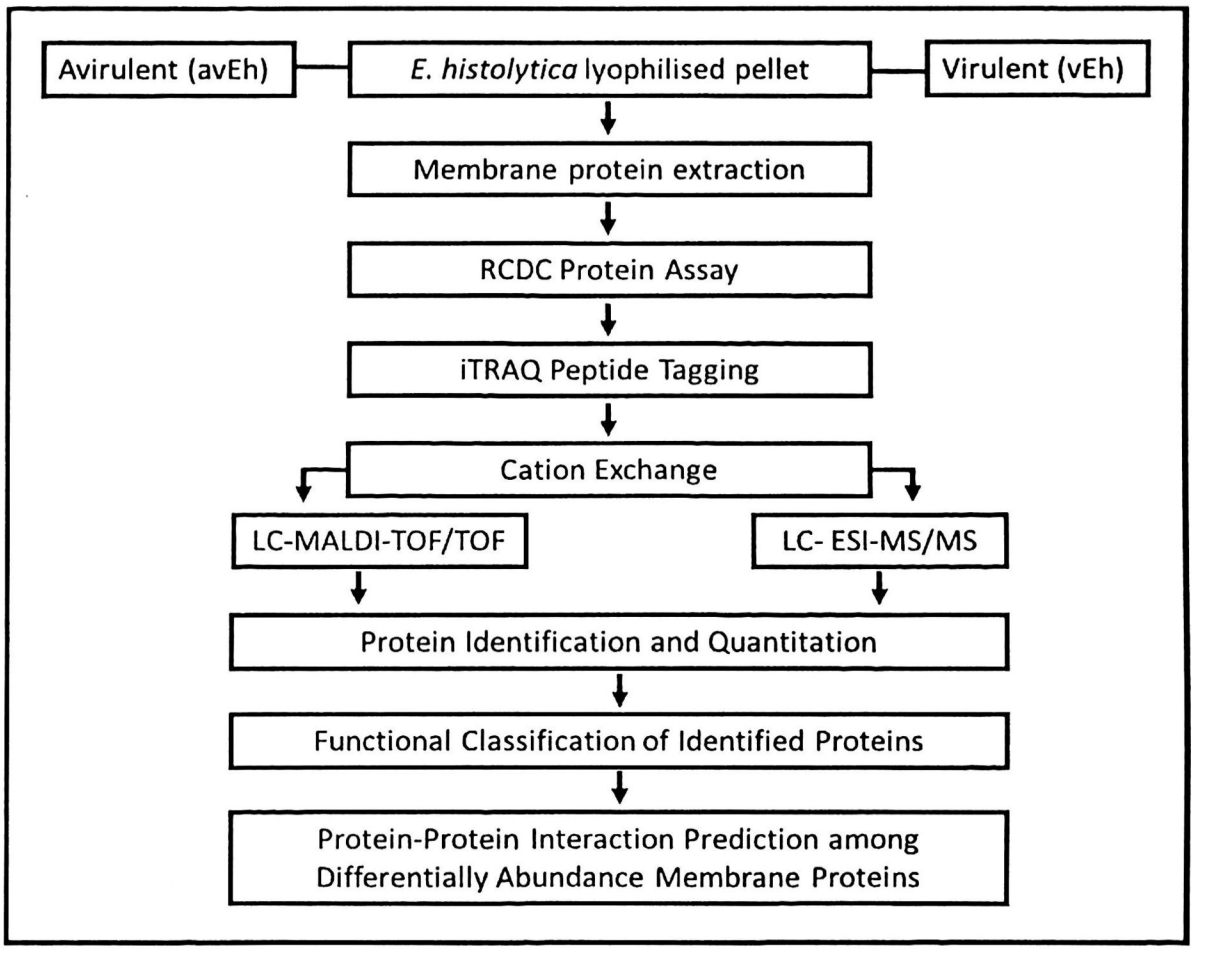

Figure 1.1 Flowchart of the study.

CHAPTER 2

LITERATURE REVIEW

2.1 Background of *Entamoeba histolytica*

E. histolytica is a unicellular eukaryotic organism that is comprised of a nucleus. The species in the genus of *Entamoeba* can be differentiated by the morphological feature in which the number of nuclei in the cysts, often can be one, four or eight nuclei (Weedall & Hall, 2011). *E. histolytica* conforms heterotrophic nutrition, where its nutrition sources are dependent upon intake from other sources to survive. This unicellular parasite is actively motile by forming the pseudopods. During phagocytosis, it also ingests food particles using the pseudopods extension.

In 1875, *E. histolytica* was initially described by a physician, Feder Lösch. The amoeba was found in faecal samples from a patient with chronic dysentery in Saint Petersburg, Russia. The pathogenicity of the *E. histolytica* cyst by oral route infection in cats was later established by Quincke and Roos (Wilmot, 1962). Fritz Schaudinn successfully clarified the non-pathogenic *Entamoeba coli* from *Entamoeba histolytica* in 1903. The name *Entamoeba histolytica* was given to this amoeba due to its ability to induce tissue destruction. In 1925, Emile Brumpt reported the existence of *E. histolytica* as a species complex, comprising two morphologically indistinguishable species, which symptomatic infection is caused by *E. dysenteriae* while *Entamoeba dispar* was found only in asymptomatic carriers (Pinilla et al., 2008; Packers, 2002; Jackson, 1998; Brumpt, 1928). The development of axenic culture medium for *E. histolytica* by Louis Diamond, in 1961 has allowed for in-depth *in vivo* and *in vitro* studies of the parasite (Diamond, 1961). In 1978, Sargeaunt and Williams applied an isoenzyme electrophoresis in the differentiation of the *E. histolytica* strains and proved that *E. histolytica* was a species complex, embracing both pathogenic and non-

pathogenic species (Sargeaunt & Williams, 1978). In 1993, the re-description of *E. histolytica* was isolated into two species: the virulent *E. histolytica* and avirulent *E. dispar* (Diamond and Clark, 1993), where the hypothesis was accepted by the World Health Organization in 1997 (Pinilla et al., 2008).

Under the taxonomy classification, *E. histolytica* was classified under the following classification: Kingdom: Protista; Subkingdom: Protozoa; Phylum: Sarcomastigophora; Subphylum: Sarcodina; Class: Lobosea; Order: Amoebida; Family: Endamoebidae; Genus: *Entamoeba*; Species: *E. histolytica*. The genomic evolution of *Entamoeba* genus provides a fascinating insight into the evolution pattern of diverse species lineages and the genetic diversity among *E. histolytica* population. The phylogeny of the *Entamoeba* genus demonstrates the presence of an evolutionary gap between *Entamoeba* species, as shown in Figure 2.1. The phylogeny tree is based upon the small subunit rRNA genes.

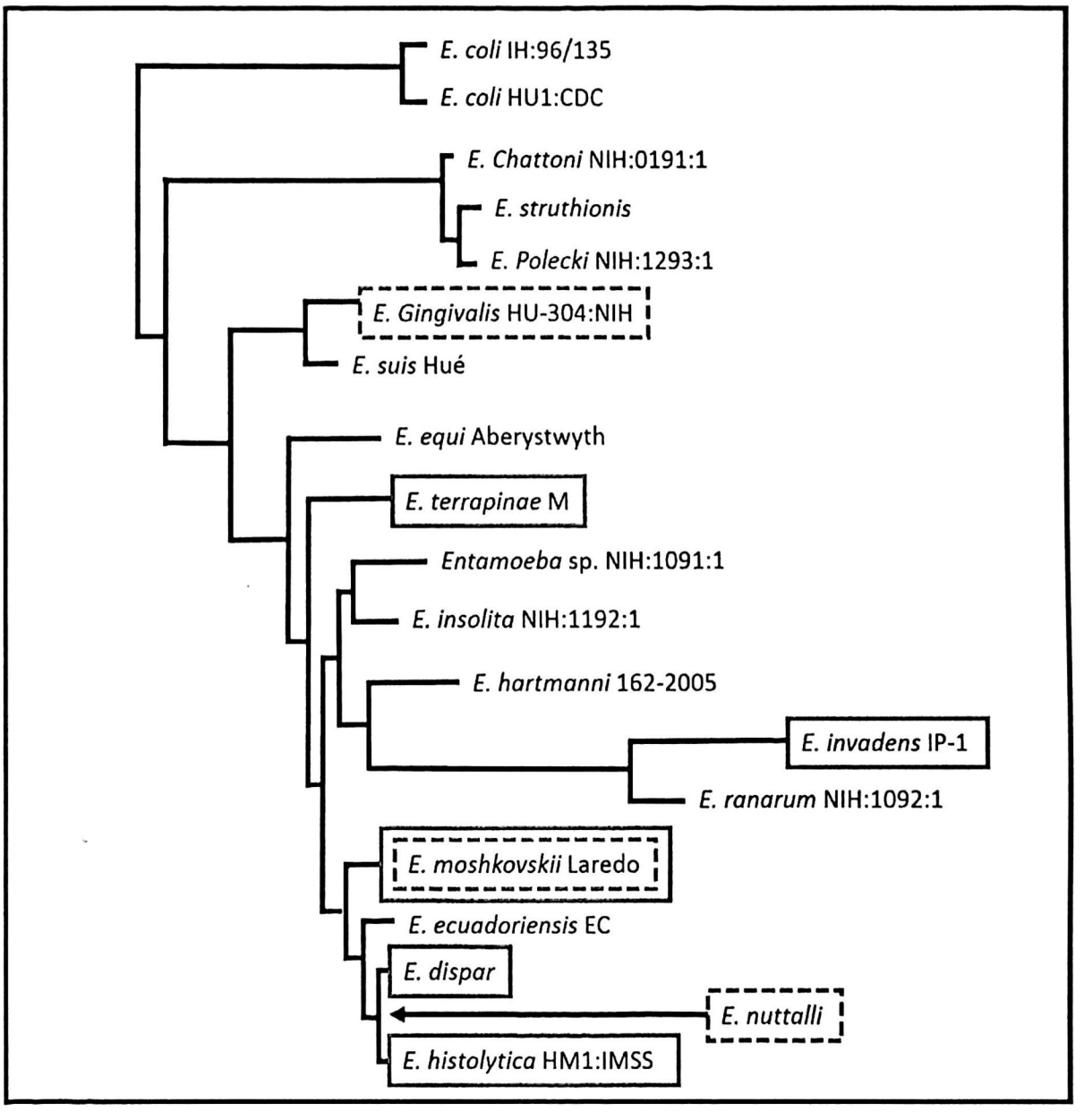

Figure 2.1 Phylogeny of *Entamoeba*, adapted from Clark et al. (2006). Full line boxes represent species where genomes have been sequenced. Dotted boxes represent species where there is poor coverage shotgun sequence data. Dashed boxes represent species due to being sequenced.

2.1.1 Life Cycle

The life cycle of *E. histolytica* is relatively simple and exists in two stages, a dormant infectious cyst stage and a vegetative invasive trophozoite stage as shown in Figure 2.2. The cysts can be present in sewage and faecally contaminated water. Ingestion cyst-contaminated food or water initiates the infection, which is the main mode of the transmission of amoebiasis (Fraust et al., 2012). The infectious cysts stay viable in the stomach and the small intestine of the host. The cyst typically has higher resistant to the harsh environmental condition and hardly destroyed. The mature cysts are able to persist viable in a damp and cool environment for several weeks outside the host. If the cysts are passed out in water, it can remain viable up to 30 days. However, the cysts are easily destroyed by desiccation, and temperatures under 5°C and over 40°C. The mature cysts also resistant to oxygen depletion, gastric acidity, and chlorination (Stenmark, 2009; Petri & Chadee, 1996). The cysts in the body will pass through the stomach and excystation occurs within the intestinal lumen. At the distal end of ileum or colon, the quadrinucleate cyst is triggered by the intestinal secretions, which results in the emergence of disease-causing, four motile metacystic trophozoites (Eichinger et al., 1997). The trophozoites multiply via binary fission in the intestine. The life cycle is completed when trophozoites undergo encystment, where the nucleus undergoes two division processes to produce a quadrinucleate cyst and excrete in the stool form or excreta.

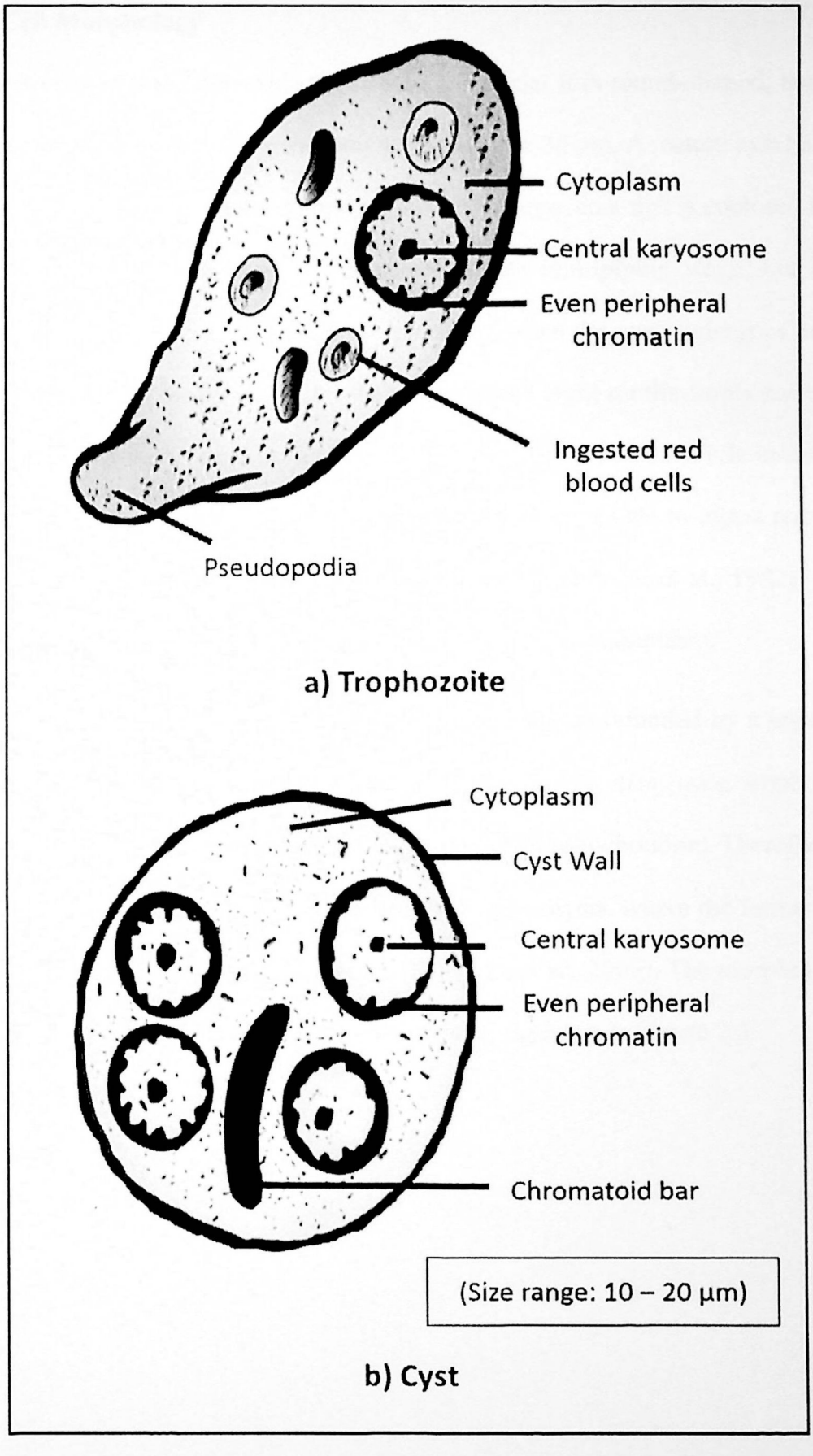

Figure 2.2 Morphological features of *E. histolytica* trophozoite and cyst (Adapted from CDC, 2017).

2.1.2 Cell Morphology

The infective cyst is the dormant stage of the life cycle. It is round-shaped, bounded by a retractile wall with the diameter ranging from 10 to 20 μm. A mature cyst has four nuclei, where each of the nuclei comprises a central karyosome that is enclosed by the peripheral chromatin. The invasive trophozoites are multiplying stage and vastly motile in their host. The trophozoites are produced when the cyst undergoes nuclear division in the small intestine. Each cyst will produce eight motile trophozoites that are pleomorphic-shaped, with the size of ranging from 10 to 60 μm. While in the large intestine, the trophozoites undergo binary fission and are capable to ingest red blood cells (Tsutsumi et al., 1992) as well as microorganisms (Bracha et al., 1982), where the phagocytised particles were not frequently found in the endoplasm.

The trophozoite also has a central karyosome that is bounded by a separating membrane with the beaded chromatin (Lohia, 2003). *E. histolytica* trophozoites possess anaerobic metabolism and lacking functional mitochondria. Therefore, the energy sources are mainly derived from anaerobic glycolysis, where the fermentation of glucose to ethanol with pyruvate occurs (Saavedra et al., 2005). The morphology of an infectious cyst and an invasive trophozoite were depicted in Figure 2.3.

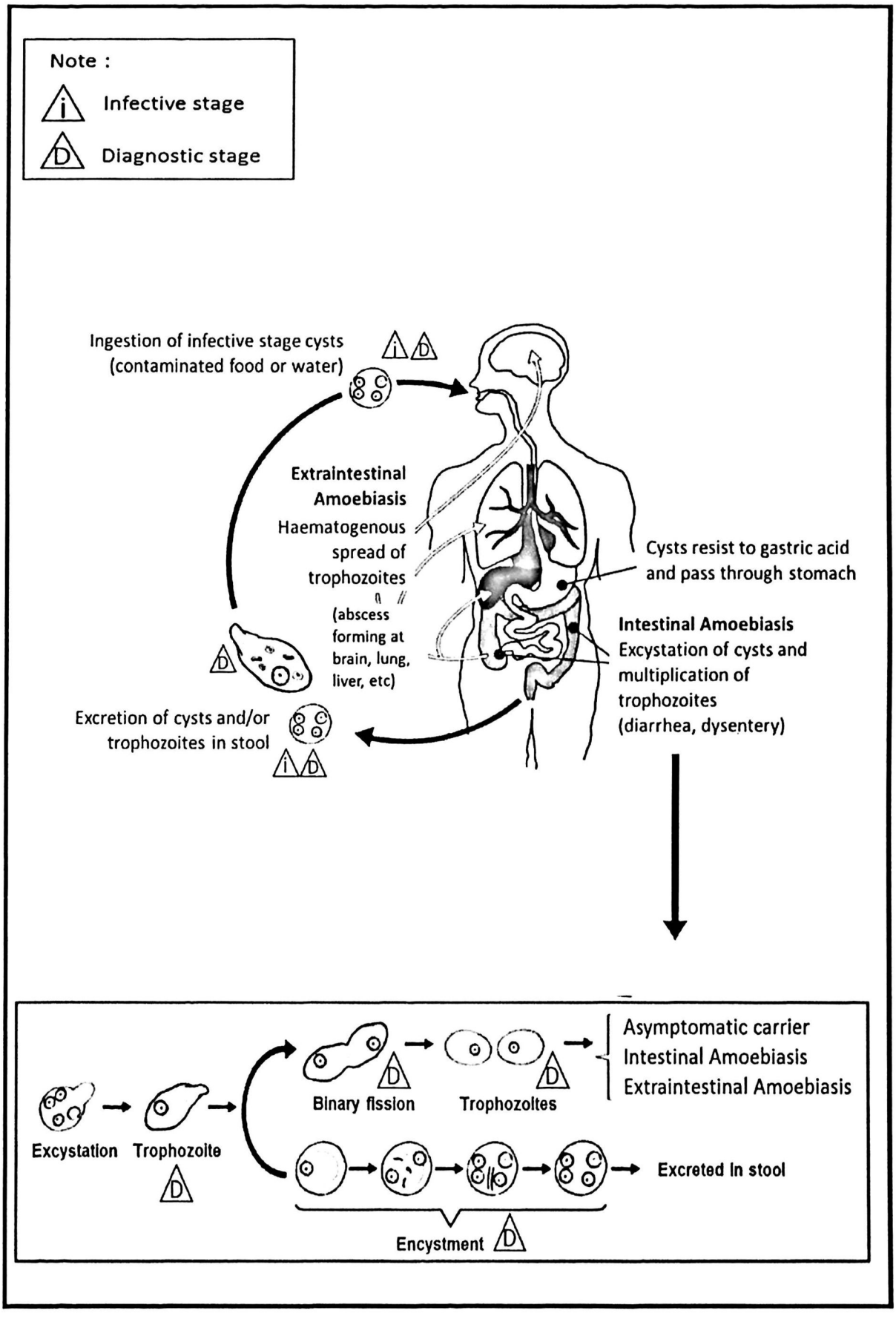

Figure 2.3 Life Cycle of *Entamoeba histolytica*. (Adapted from CDC, 2017).

2.1.3 Transmission

Human is the only host of amoebic infection since there is currently no zoonotic reservoir of *E. histolytica* (Petri et al., 2002). Furthermore, the transmission of amoebiasis does not involve any insect vectors or other parasites (Petri et al., 2002). Transmission usually occurs when there is a direct contact between an infected person and the consumption of cyst-contaminated food or water. Water source plays a vital role in the transmission of amoebiasis. In rural areas, the usage of untreated river water among the rural population is commonly practiced. Apart from that, rural communities tend to defecate in the shrubberies and nearby river from their houses. This will definitely increase the risk of water pollution and contamination of infectious cysts from human faeces mainly during the rainy season. This is because the rainwater will flow down to the ground surface through streams and the contaminated water reaches the rivers, which is unfit for consumption (Ngui et al., 2012).

A proper health education and community awareness regarding amoebiasis should be given to the communities to prevent the amoebic infection. Practical control measures among household such as appropriate water treatment should be implemented to minimize the potential risk of parasitic amoeba cyst-contaminated drinking water. For example, by educating the communities to consume boiled water instead of drinking water from river or other water sources without boiling (A WHO Meeting, 1985).

2.1.4 Epidemiology

E. histolytica infection poses a substantial health risk worldwide, where it affects 40-50 millions of people and causes 100,000 death per annum globally. The highly endemic countries that have been infected by the invasive diseases caused by *E. histolytica* include Africa (Ravdin et al., 2003; Abd-Alla et al., 2006), India and

Bangladesh (Haque et al., 2001), Southeast Asia (Blessmann et al., 2002), the Americas (Ramos et al., 2005) and Egypt (Abd-Alla et al., 2000). The highest morbidity and mortality cases were recorded in Central and South America, Africa and India (WHO, 1997). In Malaysia, several studies (Rajeswari et al., 1994; Aza et al., 2003; Norhayati et al., 2003; Hakim et al., 2007; Lim et al., 2009; Ngui et al., 2011) had reported that amoebiasis caused by *Entamoeba* species were prevalent typically in rural areas which had poor socioeconomic condition, poor environmental sanitation, and lack of personal hygiene practices compared to urban areas.

The global amoebiasis epidemiology statistics data still remains unclear due to the complication in distinguishing the pathogenic *E. histolytica*, and the non-pathogenic *E. dispar* and *E. moshkovskii*, which are morphologically similar but genetically different (Ali et al., 2008). Therefore, instead of relying on the routine diagnostic method in tropical countries such as microscopy, a more specific molecular method namely polymerase chain reaction-based approach was used to differentiate these morphologically identical species (Ali et al., 2003; Fotedar et al., 2007; Khairnar et al., 2007). On the other hand, the distribution of the disease caused by the parasite from the Africa nations was assessed through several studies with the use of microscopic examination (Alonzo et al., 1993; Molback et al., 1994; Njoya et al., 1999; Roche et al., 1999). The prevalence of individuals with parasites carrier was ranging from 6% to 75%. While in Southeast Asian countries like central Vietnam, the incident rate of ALA was recorded as high as 21 cases per 100,000 inhabitants (Blessmann et al., 2002).

Meanwhile, numerous local studies in Malaysia also have been performed to distinguish the different species of *Entamoeba* via microscopic examination and nested polymerase chain reaction (PCR) method with the overall *Entamoeba* infection

rate ranging from 17.6% to 19.5% (Anuar et al., 2012; Ngui *et al.*, 2012; Lau et al., 2013). These studies also reported that the infection caused by *E. histolytica* was the highest compared to the infection caused by *E. dispar* and *E. moshkovskii*. On the other hand, the prevalence of intestinal parasitism in rural and remote areas of West Malaysia has been demonstrated by Ngui et al. (2011), reported that the prevalence of *E. histolytica/E. dispar* was 10.2%. In another study, the prevalence of *E. histolytica* among aborigine communities in Pahang was 18.5% (Yusuf et al., 2007). Intestinal parasitism among children has also been assessed in Malaysia. For example, the prevalence of *E. histolytica* was 9.9%, and it was one of the most common parasites found among the children in Gombak (Rajeswari et al., 1994).

2.1.5 Diagnosis

There are several methods are being practiced to diagnose amoebiasis. The conventional diagnosis methods include microscopy and isoenzyme analysis. The traditional microscopic examination on patients stool sample was commonly practiced previously before the molecular techniques were established. However, this method leads to the high tendency of misinterpretation and inaccurate identification especially in early epidemiological studies (Martinez-Palomo, 1986; Gutiérrez et al., 1990), before the discovery of the morphologically similar non-pathogenic strain *E. dispar*. Meanwhile, isoenzyme analysis is a better approach compared to microscopy due to its ability to distinguish the pathogenic and non-pathogenic strains of *Entamoeba* species. There are four types of glycolytic enzymes were utilized, namely hexokinase, phosphoglucomutase, glucose-6-phosphate isomerase (GPI), and malic enzyme (Razmjou et al., 2006). The downsides of this method were time-consuming and intensive laboratory procedures. Before proceeding, this method requires four to ten days for the trophozoites to grow in the artificial media prior to starch-gel

electrophoresis. Also, the culture may unable to achieve successful growth (Haque & Petri, 2006; Ackers, 2002).

The advancement of technology leads to the introduction of various molecular diagnostic tests and the innovation of rapid diagnosis kits, which provide sensitive and specific results (Fotedar et al., 2007; Petri and Singh, 1999). The diagnostic tests include enzyme-linked immunosorbent assay (ELISA), indirect haemagglutination assay (IHA) and latex agglutination. ELISA and IHA are the most common serological techniques that have been used in laboratories for the diagnosis of amoebiasis. ELISA has high sensitivity for antibody detection while IHA method is simple to execute and is frequently practiced in many parasitology laboratories. On the other hand, the DNA-based techniques, such as real-time PCR assay is also one of the latest diagnostic tools for the detection of *E. histolytica* (Othman et al., 2010). The benefits of using real-time PCR are extremely sensitive and time-saving which benefited the researchers for the diagnostics and epidemiological assessment studies (Roy et al., 2005; Ahmad et al., 2007).

2.1.6 Treatment

The first potent tissue amoebicide was introduced in 1912, namely Emetine. However, this drug causes serious side effects such as vomiting and cardiotoxic (Knight, 1980). Alternatively, the nitroimidazole derivatives such as metronidazole, tinidazole, and ornidazole are the recommended drugs for amoebiasis treatment as they are remarkably safe when compared to emetine (Stanley, 2003). The amoebicidal properties of metronidazole were acknowledged and used as the drug for amoebiasis treatment in the mid-1960s (Upcroft et al., 2001). Furthermore, metronidazole is also commonly used to treat the infection which is caused by a wide range of anaerobic bacteria effectively due to the low-redox activating enzymes (Freeman et al., 1997).

Generally, metronidazole was commonly used in therapeutic and prophylactic for the treatment of major and minor amoebiasis that exposed to *E. histolytica.*

The treatment of amoebiasis using metronidazole often subsequent with luminal agents such as paromomycin and iodoquinol to eradicate infection, particularly for individuals that are suffered from amoebic colitis and amoebic liver abscess (Powell et al., 1969; Pehrson and Bengtsson, 1984). For the asymptomatic carrier, the infected patients are also treated with the drugs to eradicate the infection. Although metronidazole and other drugs are considered safe to use, prolonged use of the drug in the infected individuals will cause side effects for instance headache, nausea, vomiting, and abdominal discomfort (Upcroft & Upcroft, 2001). The drugs that are commercially used for the treatment of amoebiasis are listed in Table 2.1, while the adverse side effects of the drugs are tabulated in Table 2.2.

Table 2.1 Commercial drug treatment for amoebiasis (Adapted from The-Medical-Letter, 2010).

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

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

Table 2.2 Mechanism and side effect of drug treatment for amoebiasis (Adapted from Stanley, 2003).

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

2.2 Virulent Factors and Pathogenesis of *Entamoeba histolytica*

The ability of *E. histolytica* to kill host cells via a cytotoxic contact-dependent mechanism has been correlated with its virulence (Ravdin et al., 1980). The process by which the host cells are killed occurs in the sequence of adherence, cytolysis and then phagocytosis of the target cells (Ravdin & Guerrant, 1981). The carbohydrate-protein binding in which the cell surface lectin of *E. histolytica* is attached to the host galactose (Gal) and N-acetyl-D-galactosamine (GalNAc) contributes to the cytotoxicity of *E. histolytica* (Petri et al., 1987; Ravdin & Guerrant, 1981). The engagement of the trophozoites to the host colonic mucin glycoproteins is suppressed by the host Gal or GalNAc (Chadee et al., 1987) as a protective action to hinder the contact-dependent cytotoxicity by *E. histolytica* (Ravdin & Guerrant, 1981). The lectin-mucin barrier also enables mucin to neutralize the lectin in order to protect the host from the contact-dependent cytotoxicity.

Based on previous report, the pathogenicity of the *E. histolytica* has been investigated to elucidate the mechanism of disease, invasion mechanism to the host tissue, as well as to identify the components and properties that permit the parasite to evade from the host's cellular and humoral immune responses (Espinosa-Cantellano et al., 1991). The interaction between parasite and host cells is the essential key where the invasion mechanism initiates and triggers the signalling pathways in the parasite. Furthermore, the complex signals network and the interaction of this parasites with other proteins could promote the reorganization of the actin cytoskeleton that permits the adhesion of parasite to the host cell (Talamás-Rohana et al., 1988; Váazquez et al., 1995; Meza, 2000). Example of the protein that is related to the invasion mechanism such as amoebic Rho protein, is found to be involved in the actin reorganization and actin-myosin interaction that facilitates adhesion (Franco-Barraza et al., 2006).

Another example is the amoebic b-amylase, which also participates during the colon invasion (Thibeaux et al., 2013).

Other than permitting the adhesion of trophozoites to the host cells, the Gal/GalNAc lectin has been demonstrated to have a high specificity and significant effects on the encystation, phagocytosis and caspase activation in host cells (Boettner, Huston & Petri, 2002). The engagement of amoebic lectin to the host cell surface mucin glycoproteins resulting in the lectin-induced host cell calcium transients, activation of caspase and apoptosis (Petri et al., 2002). Generally, the trophozoites and the host colonic mucin are dynamically associated, where the trophozoites play roles in the induction of colonic mucin secretion and colonic mucin degradation (Chadee et al., 1988). In an *in vitro* study, the resistance of mucin-productive cell lines towards killing by *E. histolytica* was encountered by the alteration of amoebic glycoproteins. This was due to the O-glycosylation inhibitors avoided the normal glycosylation of the mucin glycoproteins and hence blocked the resistance (Petri et al., 2002).

The interaction of trophozoites with the mucin glycoproteins induced the encystation development pathway during the extracellular adhesive activity regulation by the parasite cytoskeleton (Petri et al., 2002). The amoebic actin cytoskeleton, cytochalasins have been demonstrated to dynamically participate in these sequences as an inhibiting agent (Ravdin & Guerrant, 1981; Lopez-Revilla & Cano-Mancera, 1982; Kobiler & Mirelman, 1981). Besides, the parasite cytoskeleton also plays a significant role that involved in killing by several mechanisms, including C3 intoxication of amoebic Rho, cytochalasin disruption and dominant-negative myosin II expression (Ravdin & Guerrant, 1981; Arhets et al., 1998; Godbold & Mann, 2000).

Phagocytosis plays a vital role in the cell growth and constitutes as one of the key virulence determinants in the pathogenesis of *E. histolytica* (Bracha et al., 1984). The phagocytic ability of *E. histolytica* is one of the prerequisite virulence factors, which able to damage the tissue-cultured mammalian cells *in vitro* and the development of hepatic abscesses *in vivo* (Orozco et al., 1983). The involvement of actin during phagocytosis had been also reported when *E. histolytica* initially interacted with the target cells (Bailey et al., 1985) via an amoebic surface protein, namely galactose/ N-acetylgalactosamine (Gal/GalNAc)-specific lectin (Petri et al., 2002; Mann, 2002). The engagement of trophozoites to the host cells triggered the cytoskeletal reorganization which included the F-actin polymerization, and vesicle trafficking was activated at the target-contacted site (Meza et al., 2006). However, the virulence of the trophozoites was progressively deprived in the axenic culture. In order to maintain their virulence, consistent contact with the animal host are necessary (Olivos et al., 2009).

Cysteine proteinase (CP) is recognized as one of the significant virulence factors in the pathogenesis of amoebiasis and plays a essential role in the tissue invasion, host tissues disruption, and cell-mediated immune response modulation (Reed et al., 1993; Campbell & Chadee, 1997; Que & Reed, 1997). One of the CP genes, namely CP5 has been suggested to potentially participate in the destruction of host tissues. The vital role of cysteine proteinase in the development of liver abscess in hamsters was determined and appeared to be interrelated with virulence. Previous *in vivo* studies have revealed that the effect of antisense inhibition on cysteine proteinase activity significantly diminished liver abscess formation in hamsters (Ankri et al., 1999) as well as in SCID mice (Stanley et al., 1995).