CERTIFICATE

This is to certify that the dissertation entitled

Determination of Acute and Active Amoebic Liver Abscess

is the bona fide record of research work done by

SHARNALI AHMED

during the period from September 2014 to June 2015

under my supervision.

ACKNOWLEDGEMENT

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Last but not least, I would like to thank my family and express my deepest gratitude to them for their continuous support, love and motivation. I hope that I have made them proud.

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List of Symbols, Abbreviations and acronym

%	Percentage
^{0}C	Degree Celsius
µg	Microgram
µL	Microlitre
ALA	Amoebic liver abscess
CSA	Crude soluble antigen
dH_2O	Distilled water
ELISA	Enzyme linked immunosorbent assay
et al.	*et alii* – 'and others'
x *g*	Gravity
g	Gram
Ig	Immunoglobulin
IHA	Indirect haemagglutination assay
L	Litre
mA	MiliAmpere
h	Hour
min	Minute
mL	Milliliter
mM	Milimolar
OD	Optical density
TBST	TBS-Tween 20
PBS	Phosphate Buffered Saline
PBST	PBS-Tween 20
PCR	Polymerase Chain Reaction
psi	Pound-force per square inch
TMB	3,3',5,5'-Tetramethylbenzidine
rpm	Revolutions per minute
COV	Cut-Off Value

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Penentuan Abses Hati Ameba Akut dan Aktif

ABSTRAK

Abses hati ameba (ALA) ialah jangkitan oleh protozoa *Entamoeba histolytica* yang boleh membawa maut. Dalam Hospital Universiti Sains Malaysia, antara ujian yang diguna untuk diagnosis ALA termasuk pengesanan antibodi menerusi kit komersial yang menggunakan antigen larut mentah (CSA) *E. histolytica*. Walau bagaimanapun, penggunaan kaedah ini hanya berfaedah di kawasan bukan endemik sebab penggunaannya di kawasan endemik sering tidak dapat membezakan jangkitan ALA semasa daripada jangkitan ALA lama. Teknik berasaskan DNA seperti *real-time PCR* adalah sensitive dan spesifik untuk mengesan DNA *E. histolytica* pada sampel biopsi hati. Walau bagaimanapun, kos yang tinggi, keperluan peralatan khusus dan kakitangan yang terlatih menghalang penggunaannya di kebanyakan tempat endemik. Kajian ini bertujuan untuk mengenal pasti jangkitan ALA akut and aktif dengan menggunakan CSA untuk masing-masing mengesan IgM dan IgG_4 di dalam serum sampel ALA yang telah dikesan menerusi kit komersial IHA. Dalam ekpserimen ini, tiga jenis asai imunojerapan berpaut enzim (ELISA) telah dibangunkan, iaitu CSA-IgG ELISA, CSA-IgG_4 ELISA and CSA-IgM ELISA. ELISA tersebut telah berjaya dibangunkan dengan mengoptimumkan parameter seperti kepekatan CSA, antibodi pertama (IgG, IgG_4 dan IgM) dan antibodi kedua (anti-IgG, anti-IgG_4 dan anti-IgM). Sensitiviti dan spesifisiti setiap ELISA yang telah dibangunkan dibandingkan dengan 30 sampel serum yang positif ALA dan 30 sampel negatif ALA telah disahkan oleh HUSM. CSA-IgG ELISA didapati mempunyai sensitiviti dan spesifisiti 96.7%; CSA-IgG_4 ELISA masing-masing mempunyai sensitiviti dan spesifisiti 83.3% dan 90.0%; dan CSA-IgM ELISA mempunyai sensitiviti 70.0% dan spesifisiti sebanyak 50.0%. Kesimpulannya, kajian ini mungkin telah

bejaya mengesan 50.0% kes ALA akut daripada jumlah kes ALA yang dikesan menerusi asai IHA; dan 83.3% kes ALA adalah jangkitan aktif.

Determination of Acute and Active Amoebic Liver Abscess

ABSTRACT

Amoebic liver abscess (ALA) is a deadly infection caused by the protozoa *Entamoeba histolytica*. At Hospital Universiti Sains Malaysia, diagnosis of ALA includes antibody detection using a commercial kit based on crude soluble antigens (CSA) of *Entamoeba histolytica*. However, this method may only be useful in non-endemic area and often cannot differentiate current and past infection when used in endemic areas. DNA-based techniques such as real-time PCR has been utilised to detect *E. histolytica* DNA of liver biopsy samples with high sensitivity and specificity, but the cost to perform this technique is high and can only be performed in laboratories where specialised equipment and trained personnel are available. This study aimed to identify acute and active infection of ALA by utilising crude soluble antigen (CSA) of *E. histotyica* to detect IgM and IgG4 respectively in ALA serum samples detected with the commercial IHA kit which detects total IgG. In this experiment, three types of enzyme-linked immunosorbent assay (ELISA) were developed, namely CSA-IgG ELISA, CSA-IgG_4 ELISA and CSA-IgM ELISA. The ELISAs were successfully developed based on optimisation of parameters such as CSA concentration, first antibody (IgG, IgG_4 dan IgM) and secondary antibody (anti-IgG, anti-IgG_4 dan anti-IgM). The sensitivity of each ELISA was compared with the 30 positive ALA serum samples and 30 negative ALA serum samples that were confirmed by IHA. CSA-IgG ELISA showed a sensitivity and specificity of 96.7%; CSA-IgG_4 ELISA revealed a sensitivity and specificity rates of 83.3% dan 90.0% respectively; and CSA-IgM ELISA were found to be 70.0% specific and 50.0% sensitive. In conclusion, this study revealed that 50.0% of the IHA confirmed ALA cases were probably acutely infected; and 83.3% of the confirmed ALA cases were probably active ALA cases.

CHAPTER ONE

INTRODUCTION

1.1 Introduction to *Entamoeba histolytica*

Entamoeba histolytica is an intestinal protozoan parasite. It is an invasive parasite causing diarrhoea, dysentery, and liver abscess in human. It belongs to the genus *Entamoeba* which has many species. Six of these *Entamoeba* species reside in the human intestinal lumen – *Entamoeba histolytica, Entamoeba dispar, Entamoeba moshkovskii, Entamoeba polecki, Entamoeba coli* and *Entamoeba hartmanni* (Tengku S.A & Norhayati M, 2011). Among these species, *E. histolytica* is the only species that is considered as pathogenic. Before the development of molecular diagnostics, it was difficult to differentiate *E.histolytica* and *E. dispar* via microscopy. Also, recent studies showed that *E. moshkovskii* has also been detected in individuals living in endemic areas of amoebiasis. Advances in molecular technology recently have allowed for clear characterization of the *Entamoeba* species.

E. histolytica can be found world-wide, but like any other intestinal protozoa it is more common in the tropical and sub-tropical zone than the temperate zone, more in developing countries with poor sanitary conditions. Previously all the deaths so far were suspected of due to the invasion of *E. histolytica*. However, the data on the incidence of *E. histolytica* now is considered overestimated since the data was collected before the differentiation of *E. histolytica* and *E. dispar* (Diamond & Clark, 1993). Also, presence of *E. moshkovskii*, which is morphologically identical to *E. histolytica* but genetically different, being detected in endemic areas of amoebiasis has made things complex. This is because previously most of the cases of amoebiasis were diagnosed using microscope – the most

common diagnostic tool, especially in developing countries (Ali *et al.*, 2003; Parija & Khairnar, 2005; Khairnar *et al.*, 2007; Fotedar *et al.*, 2008; Tengku Shahrul Anuar *et al.*, 2012).

Global statistics on the prevalence of *E. histolytica* infection indicates that 90% of the infected individuals remain asymptomatic while the other 10% develop clinically overt disease (Jackson *et al.*, 1985; Haque *et al.*, 1999). Nevertheless, it still causes high morbidity and mortality rates in many countries (e.g. Bangladesh, India, countries in western Africa).

1.2 Life Cycle of *Entamoeba histolytica*

Humans are the only host for *E. histolytica*. There are two forms of *E. histolytica*, namely trophozoite form and cyst form. The trophozoite form is the motile and the multiplying form. The cyst is the dormant and infective form. The cyst is resistant to extremes of temperatures, pH and other environmental changes (e.g. dry/humid). The trophozoite on the other hand is quite fragile. It can survive inside human but will die within minutes after coming out of the human body.

When the cysts (infective form) are swallowed along with the contaminated food and drinks by a susceptible person, they develop further inside the gut. The cysts pass though the stomach. They are resistant to gastric juice but the walls of the cysts will then be digested by trypsin in the duodenum. Then in the lower part of ileum excystation occurs, where the cysts turn into trophozoites. The trophozoites then reside in the large intestine, where they

multiply. Some of the trophozoites will try to penetrate through the intestinal wall and travel to other parts of the body, mainly the liver. Some of the trophozoites transform into quadrinucleate cysts again (encystations), which are then passed out through faeces. Both the trophozoites and cysts are passed out, but trophozoites die whereas the cysts survive. The cysts are then ingested again by another individual through contaminated food and water. The infected person (asymptomatic) without knowing passes on stool containing cysts, and the vicious cycle goes on, infecting hundreds and thousands of people over time.

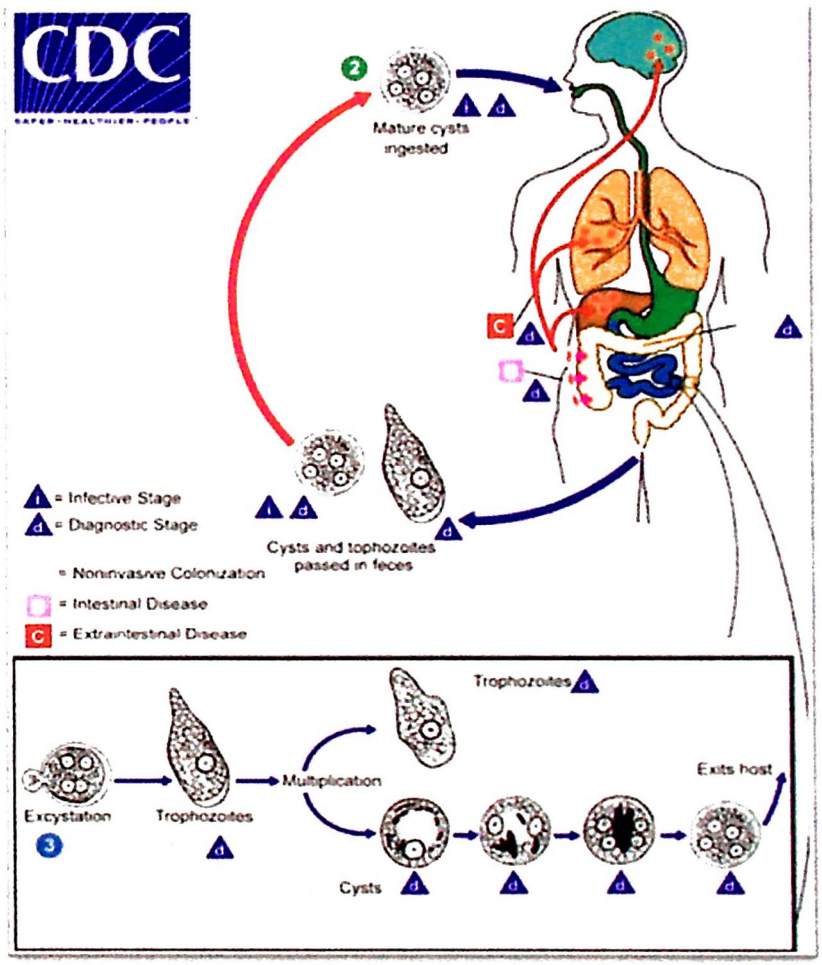

Figure1.1: Life Cycle of *E. histolytica*

(http://upload.wikimedia.org/wikipedia/commons/0/0a/Entamoeba_histolytica_Amebiasis_LifeCycle.gif)

1.3 Epidemiology of Amoebiasis

The epidemiology of amoebiasis has significantly changed since the differentiation of *E. histolytica* and *E. dispar*. According to World Health Organization, WHO (1997), around 10% of the world population get infected with the parasite, and about 2% of the infected population develop the invasive from of disease. Despite the small percentage, about 100,000 people die each year due to medical complications of invasive amoebiasis. It is the second leading cause of death from parasitic diseases, with malaria being first. However, there are errors to the given figure considering the differentiation of *E. histolytica* and *E. dispar* later on; the presence of non-pathogenic *E. moshkovskii* in endemic regions recently also alters the calculations. However, the recent available prevalence and morbidity data obtained through molecular techniques has allowed the construction of a more reliable map of endemic regions all over the world [the Asian subcontinent (Bangladesh, India), Africa, Asian Pacific Countries (Thailand, Japan), South and Central America (Colombia, Mexico)].

Epidemiological studies also mentioned the importance of host factors in *E. histolytica* infection, especially when the re-infection rates in a study population found were about 2.7X higher when compared with the primary infection rates. This suggested either a short-lived immunity or genetic susceptibility of the host (Blessmann *et al.*, 2003). Serum IgG was screened for *E. histolytica* to confirm that immune responses were clustered in families (Haque *et al.*, 2002) and presently, protection against the *E. histolytica* infection is thought to be genetically linked to HLA class II-restricted immune response.

1.4 Pathogenesis of *E. histolytica*

Scientists have previously tried to perform *in vivo* study of the pathogenesis of amoebiasis in a human model. They have tried to carry out amoebiasis in cell line, but it was a failure as the trophozoites ended up breaking the cells. After that scientists tried carrying out the pathogenesis of ALA in animal model. Hamsters are used more often to understand the pathogenesis of amoebiasis and to look deeper into the formation of ALA.

The pathogenesis associated with *E. histolytica* ranges from non-invasive intestinal disease to extra-intestinal disease. The non-invasive disease is often asymptomatic, but can cause diarrhoea and other gastrointestinal problems like abdominal pains and cramps. Most of this type of manifestation will usually resolve on its own within a month. The invasive disease can become progressively worse and can develop to a more serious type of disease.

The pathological range of amoebic colitis starts from the mucosal thickening, multiple discrete ulcers separated by regions of colonic mucosa of normal appearance, inflames and oedematous mucosa, necrosis and perforation of the intestinal wall.

It begins with *E. histolytica* tophozoites adhering to the colonic epithelial cells, through either galactose or N-acetylgalactosamine specific lectin. Mammalian cell without N-terminal galactose or N-acetylgalactosamine residues are resistant to adherence and attack by the amoebic trophozoites as cell to cell contact is needed by amoeba in order to break down the cell.

Cytolytic properties of *E. histolytica* were discovered long ago but their composition and function at molecular level were unknown. A family of molecules known as amoebopores contains at least three small peptides which are capable of forming holes in the lipid bilayers of the cells. Human cells coming in contact with trophozoites can be destroyed within seconds because of the amoebapores. These molecules have similar structure to granulysins and NK-lysins produced by the mammalian T-cells.

E. histolytica can also kill mammalian cells by induction of apoptosis. The exact reason for amoebic trophozoites being able to cause apoptosis is still being investigated. Experiment on animals showed that hepatocytes require activation by caspase.

Amoebic invasion through the mucosa and into the submucosal tissue is characteristic of amoebic colitis. Lateral extension through submucosal tissue gives rise to the classic 'flask-shaped' ulcer of amoebiasis. Contact between trophozoites and extracellular matrix protein fibronectin can trigger signalling cascades within the parasite, which can alter the arrangement of actine, thus altering the adherence and motility.

There are strong evidences of cysteine proteinases being one of the virulence factors as well. Cysteine proteinases are proteolytic enzymes secreted by the parasite which move to the mucus and epithelial barrier, thus facilitating the inside the tissue. These enzymes together with some unknown factors lead to the formation of ulcer and consequently the migration of amoebic trophozoites to the liver.

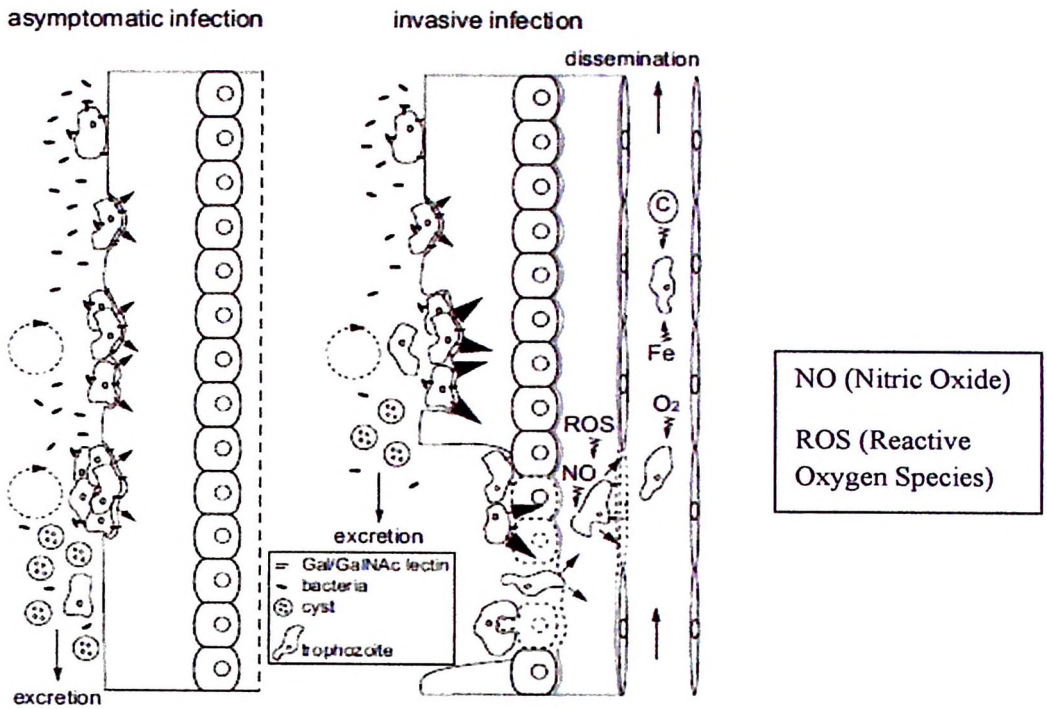

Figure 1.2: Trophozoites residing and invading in the lumen (Faust & Guillen, 2012)

Table1.1: *E. histolytica* processes and factors contributing to virulence (Faust & Guillen, 2012)

Motility						
Actin	Actin-BPs	Myosin IB, Myosin II	Rho, Rac small GTPases	CaBP1		
Adherence						
Gal/GalNAc lectin	KERP1	B1FNR	ADH112	CP-A5 (RGD)	STIRP	M17
Cytotoxicity, host cell killing						
CPs	APs	PLs	CPADH	ROM 1 Protease		
Phagocytosis						
CaBP1/C2PK	TMKs	Myosin IB, Myosin II	Rab, Rac small GTPases	PAK	SREHP	FP4
Vessicle trafficking						
Vps4	Arf	CaBPs	Rab small GTPase			
Host defence escape						
CPs	Peroxiredoxin	PPGs	Arginase	serp	MLIF	PGE2
Stress response						
Hsps	Peroxiredoxin	Ruberythrin	Thioredoxin reductase	FeSOD	p34	
Glucose starvation response						
KriP1	LgL1	URE3-BP				
Calcium signalling						
CaBP1/C2PK	URE3-BP					

BPs, binding proteins; CaBP, calcium-binding protein; b1FNR, beta-1 integrin-like fibronectin receptor; ADH, adhesin; STIRP, serine threonine isoleucine-rich protein; M17, immunodominant antigen M17; ROM, rhomboid; PL, phospholipase; CPADH, complex of CP112 and ADH112; C2PK, C2 domain-containing protein kinase; TMK, transmembrane kinase; PAK, p21-activated kinase; SREHP, serine-rich E. histolytica protein; FP, FYVE-domain-containing protein; Vps, vacuolar protein sorting; serp, serpin (serine protease inhibitor); Arf, ADP-ribosylation factor GTPase; MLIF, monocyte locomotion inhibitor factor; PGE2, prostaglandin E2; FeSOD, iron-superoxide dismutase; p34, NADPH:flavin oxidoreductase; KRiP, lysine-rich protein; LgL, light subunit of Gal/GalNAc lectin; URE3-BP, upstream regulatory element 3 binding protein.

1.5 Treatment

Metronidazole is still the ideal treatment to treat patients suffering from amoebiasis. It is metabolized in the liver, and the simultaneous administration of the drug that increase or decrease the microsomal liver enzyme activity may lead to altered plasma concentrations. The major route of elimination for metronidazole and its metabolites is urine, with a minor part being excreted via faeces. Metronidazole is used for the treatment of both the anaerobic protozoa and also anaerobic bacteria (Upcroft & Upcroft, 2001).

It enters the cell as a prodrug by passive diffusion and is activated either in the cytoplasm or in specific organelles in the protozoa. Metronidazole molecule is converted to a short lived nitrosol free radical by intracellular reduction. This includes the transfer of an electron into the ntiro group of the drug. This form of drug is cytotoxic and can interact with DNA molecule. The actual mechanism of action of metronidazole is still unclear but includes the inhibition of DNA synthesis and DNA damage by oxidation, causing single-stranded and double strand breaks which lead to DNA degradation and thus cell death.

Oral doses of metronidazole are easily absorbed and can be found in most body fluids with very few side effects. Metronidazole was introduced in 1960s, and is still being used widely, both therapeutically and prophylactically, to treat both major and minor cases of infections caused by *Entamoeba*. Inappropriate short-term exposure and exposure to sublethal levels of metronidazole are usually prescribed for prophylaxis and are precisely the conditions under which drug resistance is induced.

1.6 Vaccination

E. histolytica is distributed worldwide and is generally associated with poor sanitary and socioeconomic conditions. Majority of the developing countries cannot afford improvements in sanitary conditions. Therefore amoebiasis at present is poorly controlled. Despite having cheap and effective therapy to treat amoebiasis, morbidity and mortality rate associated with invasive amoebic infection is still high. Since humans are the only hosts of *E. histolytica*, an appropriate program should be able to eradicate amoebiasis. A number of amoeba proteins have been tested as possible vaccine candidates and some of the protein molecules have been found to be effective in experiments carried out in animals (mainly in hamster and mouse).

N-acetyl galactosamine-inhibitable amoeba lectin (Gal/GalNAc) is one of the candidate vaccine antigens which has met all the basic requirements as a subunit vaccine candidate. The lectin plays an important role in adherence of trophozoites to the host cells and also in cytotoxicity. Experiments carried out in mouse have shown that the use of Gal/GalNAc lectin used as a heavy subunit of a vaccine has prevented formation of amoebic colitis and amoebic liver abscess (Petri, 2006).

Serine-Rich *E. histolytica* Protein (SREHP) is the second candidate of vaccine antigens. SREHP acts as a chemoattractant to *E. histolytica* trophozoites and mediates the binding of trophozoites of *E. histolytica* to the host cells. Studies were carried out in hamsters in which hamsters were injected intraperitoneally with SREHP as a subunit of the

recombinant antigen. Studies showed that over 80% of the hamsters did not develop amoebiasis later on (Parija, 2011).

Other candidates for development of vaccination are peroxiredoxins, lipophophosphoglycans (LPGs), and cysteine proteinases. The production of these molecules at a large scale is rather difficult compared to the production of Gal/GalNAc lectin and SREHP. Therefore the formation of recombinant antigens using these molecules will take a long time.

Overall, the development of vaccines to prevent amoebiasis is still at its infancy. It will take years to produce the perfect recombinant antigen, to determine the dosage of it in humans to induce immunity, its safety, toxicity and side-effects in human.

1.7 Diagnosis

1.7.1 Current Diagnosis

The laboratory tests used at present to diagnose amoebiasis are microscopy, culture, ultrasound, biopsy, antigen detection, serology tests and polymerase chain reaction. Microscopic examination of stool to detect trophozoites is maximum 50% sensitive from a stool sample in amoebic colitis. Generally, stool examinations of patients suffering from amoebic liver abscess are negative. It is recommended by World Health Organisation that *E. histolytica* should be specifically identified and if present, the patient must be treated immediately. Treatment is unnecessary if only *E. dispar* or *E. moshkovskii* is identified (WHO 1997).

Historically light microscopy has been the method of choice to diagnose amoebiasis which relies on identifying cysts or trophozoites of *E. histolytica*. The presence of amoebic trophozoites containing ingested Red Blood Cells (RBCs) strongly support *E. histolytica* infection. It is difficult to find the trophozoites as the sensitivity is very low. It is also very difficult to distinguish between *E. histolytica* trophozoites and trophozoites of other non-pathogenic *Entamoeba* species. Another problem is that the trophozoites of *Entamoeba* species are also difficult to distinguish from macrophages.

Culture of *Entamoeba* species in selective media was carried out as well in order to differentiate between the pathogenic and non-pathogenic species. However, the culture methods are time-consuming, laborious and often are unrewarding as the sensitivity is very low.

Antigen detection tests and serology assays are now available to diagnose amoebiasis. The most common assay used to detect and measure serum anti-lectin antibody is ELISA (Enzyme Linked Immunosorbent Assay). In detection of antibodies in patients with amoebic liver abscess (ALA), the sensitivity and specificity of ELISA is 97.9% and 94.8%. Immunofluorescent assay (IFA) can be used also as it is quick and reliable. Immunoelectrophoresis, counter-immunoelectrophoresis (CIE), and immunodiffusion tests can also be used but all of them have different sensitivity and specificity and it is difficult to detect between recent and past infection.

The use of Polymerase Chain Reaction (PCR) to specifically amplify minute amounts of pathogenic DNA has been useful. Several real-time PCRs have been designed to detect and to identify the pathogenic stains of *E. histolytica*.

1.7.2 Problem in Diagnosis of *E. histolytica* Infection

In majority of the endemic areas, microscopic examination is still the primary method of diagnosis. Microscopic examination has maximum 50% accuracy, and is unable to differentiate between *E. histolytica* and *E. dispar*. Ultrasound can be used to detect liver abscess but cannot be identified whether it is pyogenic abscess (bacterial) or amoebic. Drainage of puss and examination of biopsy can be used to determine ALA, but both the techniques are invasive and require great deal of technical knowledge and skill.

Serology assays are quite sensitive and specific (around 90%) in diagnosing extra-intestinal amoebiasis. However, using serology assay only it is difficult to determine the whether it is early or late stage of infection, as antibodies against *E. histolytica* will remain in the body for a long time after the infection. For serological assays, kits and reagents need to be bought from companies which are quite expensive. PCR and real-time PCR are quite accurate in detecting *E. histolytica* but it is very expensive.

Previously studies were carried out in designing in-house indirect ELISA (Tan *et al.*, 2013) to diagnose ALA. Although the readings were quite accurate, chances of having false-positive result is high considering the fact that antibodies remain in human body for years after the infection has occurred (Fotedar *et al.*, 2007).

1.8 Rationale of the study

1.8.1 Previous Work on This Project

Previously, *E. histolytica* was cultured axenically and then used to infect the liver of hamsters. Then recombinant antigen was expressed, purified and used in ELISA. For the development of in-house ELISAs, in most of the cases recombinant antigens were used. These recombinant antigens, although quite specific, take longer time in preparing compared to crude soluble antigen (CSA).

For this study, CSA was prepared and the principle of indirect ELISA was used to determine the titre of IgG and IgG_4 antibodies to determine acute and active ALA in human.

The sera samples were obtained from Serum Bank of Microbiology and Parasitology Department, School of Medical Science, Universiti Sains Malaysia. The positive ALA serum samples were collected from patients diagnosed with ALA upon admission to Hospital Universiti Sains Malaysia (HUSM). Besides the other clinical observations, all the positive serum samples were tested positive by IHA kit.

Previous experiments using ELISA have shown that the level of IgG1 and IgG4 were relatively high in patients with amoebiasis. The sensitivity and specificity of the test in patients with ALA ranged from 74.7% - 100% and 91% - 97% (Nath *et al*, 2013).

1.9 Objectives of the Study

General Objective –

- To Develop CSA/IgG-ELISA, CSA/IgM-ELISA and CSA/IgG_4-ELISA

Specific Objectives -

1) To compare the efficiency of the in-house CSA/IgG-ELISA with the commercial IHA kit for detection of human ALA.
2) To determine the acute ALA serum by CSA/IgM-ELISA.
3) To determine the active ALA serum samples by CSA/IgG_4-ELISA.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Optimization of Crude Soluble Antigen (CSA)

2.1.1. Culture of *E. histolytica*

E. histolytica was cultured in Tripticase-Yeast Extract-Iron and Serum Medium (TSI-S-33) at 36°C with 80% filled medium. The medium was changed for every 48-72 hours. For routine maintenance, the trophozoites were cultured in Nunc slant-end culture tube (13 cm^3) filled with about 10 ml of culture medium. For culture of trophozoites at large scale, the cells were cultured in Nunc culture flask (25 cm^3) with about 46 ml of culture medium.

2.1.2. Maintenance of *E. histolytica* culture

A culture tube was observed under a microscope for growth of the trophozoites. The tube was inverted repeatedly about 5 times to mix the unattached cells. Under sterile conditions, the screw cap of the tube was flamed with a Bunsen burner for a few times. The mouth of the tube was wiped with sterile gauze. The old medium was then discarded into a beaker containing 10% sodium hypochlorite (Chlorox, Malaysia). Then the tube was refilled with about 8 ml of fresh TYI-S-33 medium with the aid of a serological pipette. The mouth of the tube was wiped with sterile gauze followed by flaming with Bunsen burner before the cap was screwed. The cap was sealed with parafilm and the tube was incubated at 36°C for 48-72 hours.

For sub-culturing or expanding or the trophozoites, a culture tube with confluent growth of trophozoites was chilled on ice for 5 minutes and gently inverted repeatedly for about 5-6 times to mix the unattached cells. Half the volume of the cell suspension was

transferred into the new culture tubes, closed and incubated at 36°C for 5 minutess for attachment of the trophozoites. Then the old media in the tubes were discarded and added with fresh culture medium. The culture tubes were incubated at 36°C for 48-74 hours.

2.1.3. Cell Harvesting

The culture flasks were incubated on ice for about 20 minutes or 5 minutes, respectively. The flasks were then inverted repeatedly for five times to mix the cells and cell suspensions were pooled into a centrifuge tube and spun at 440 × *g* for 2 mins to obtain the cell pellet. Subsequently, the supernatant was discarded; the pellet was resuspended with sterile PBS(A) and was centrifuged again. The process of centrifugation, discarding of supernatant and resuspension of cells with sterile PBS(A) were carried out for three times to wash the cells. After washing for the last time, the cell pellet was resuspended with PBS(A) and counted. The cells suspension was aliquoted to 10 $\times 10^6$ cells per micro-centrifuge tube, followed by centrifugation to discard the supernatant. Lastly, the micro-centrifuge tubes containing the cells pellet were labelled and stored at -80°C until used.

2.1.4. Cell Counting

Cell counting was performed using Trypan blue exclusion method using a Neubauer's chamber. The dead cells and background were stained blue, while the viable cells remained unstained. Upon collection of cell suspension in centrifuge tube, the tube was chilled on ice for 2 min to prevent attachment of cells onto the tube wall. The cells suspension was resuspended, and then 50 µl of the suspension was mixed with 50 µl of 0.4% Trypan blue. 10 µl of the mixture was pipette into the Neubauer's chamber. Duplicate counting was performed with the aid of tally counter. Figure 2.1 shows the schematic diagram of counting cell area and the formula to deduce the cell density of the cell suspension.

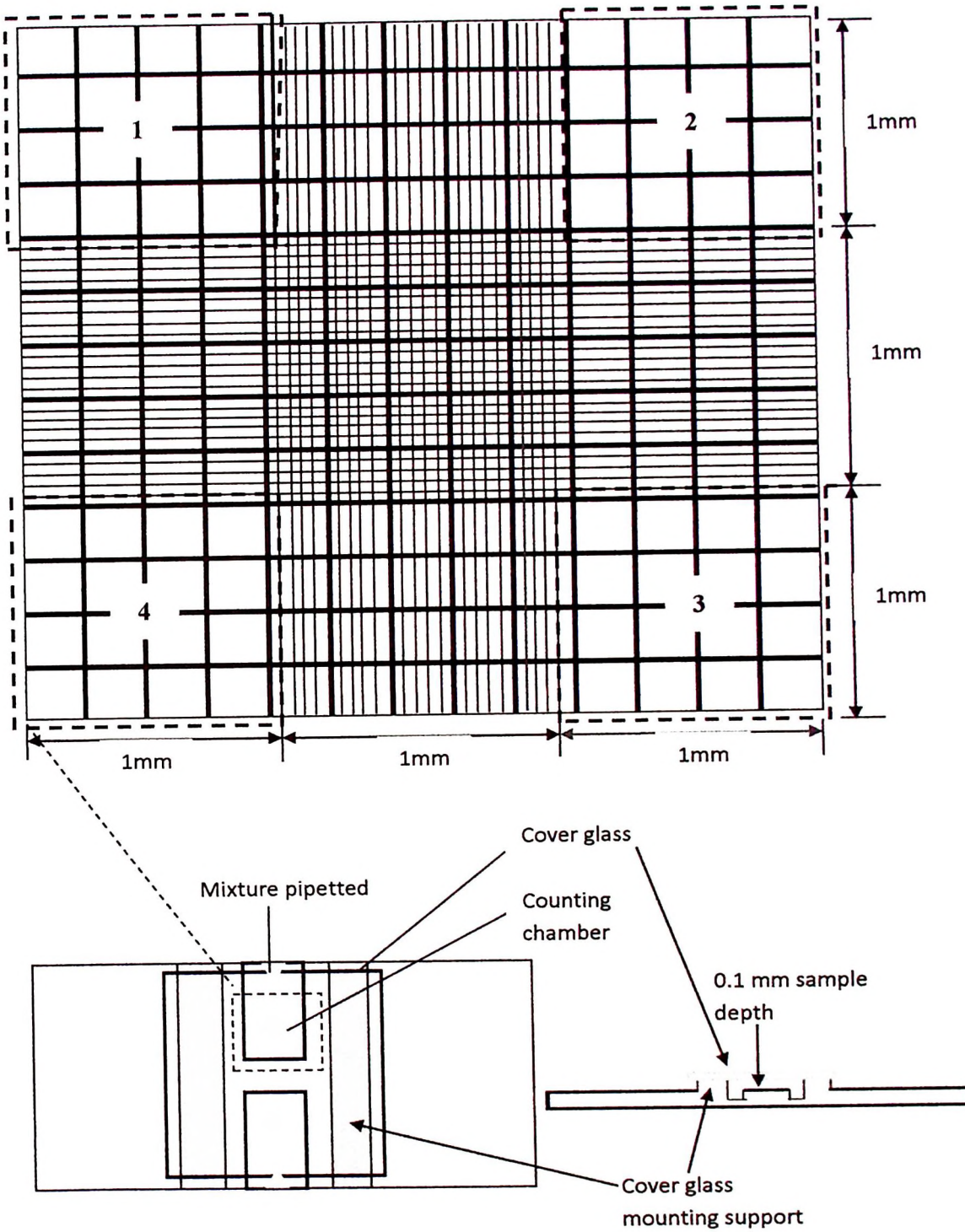

Figure 2.1: Schematic diagram of cells counting areas in Neubauer's chamber

Formula

Cell density for amoeba cells

$= N / V \times DF$

$= N / (4 \times 10^{-4}) \times 2$

$= N \times 5000$

N = Number of cells in four counting areas

V = volume of the four counting areas

$= 4 \times (0.1 \text{ mm} \times 1 \text{ mm} \times 1\text{mm})$

$= 0.4 \times 1 \text{ mm}^3$

$= 4 \times 10^{-4} \text{ cm}^3$

$= 4 \times 10^{-4} \text{ ml}$

DF = Dilution factor of the medium

(e.g. DF of 1 part cell suspension with 1 part Trypan blue = 2)

Cell viability

$$= \frac{Number\ of\ viable\ cells\ in\ four\ counting\ areas}{Total\ number\ of\ cells\ (viable\ and\ dead) in\ four\ counting\ areas} \times 100\%$$

2.1.5 Preparation of Crude Soluble Antigen (CSA)

In a centrifuge tube consisting of about 10 M (10 x 10^6) cells, 400 µl of Lysis Buffer was added along with 20 µl of 0.5 M iodoacetamide. The cell lysate was subjected to sonification at 10% amplitude for 1 minute of sonification with 0.5 second pulse-on and 0.5 second pulse-off on crushed ice. Total of three sonification cycles were carried out and in between each cycle, the cell lysate was mixed using pipette in order to prevent sedimentation. The cell lysate was then centrifuged at 10,000 *g*, at 4°C for 10 minutes. The supernatant was then collected and stored at -80°C.

2.1.6 Measuring of Protein (CSA) Concentration

About 1 ml of protein assay solution (Bradford protein concentration) and 20 µl of supernatant were put in a cuvette and the concentration of protein was measured. The CSA concentration determined was 7.0 mg/ml.

2.1.7 Dilution of Crude Soluble Antigen (CSA)

A volume of 11.4 µl of the antigen was dissolved in 2 ml of Phosphate Buffer. Then in a separate ELISA plate, in the first row, 250 µl of antigen solution was added. In the other rows, 125 µl of Phosphate buffer solution was added. Then from the first row, using a multipipetter, 125 µl of antigen solution was taken and put in the second row, mixing it at least 4-5 times and then transferring into the third row again and mixing, going on until the last row, diluting the concentration (2X every time, 1.75 mg/ml, 0.875.....0.014) of antigen for each row, which was used in ELISA later on.

2.1.8 Enzyme Linked Immunosorbent Assay (ELISA) – To Determine the Ideal Concentration of CSA to be used for Coating

First, 100 µl of antigen solution (CSA) was added to each well in an ELISA plate and was incubated overnight at 4°C in a moist chamber.

After overnight incubation, the plate was incubated at room temperature (25°C) for 1 hour on a rotator at 200 rpm (rotation per minute). The plate was then washed with 200 µl of Tween-20 dissolved in 1X Phosphate buffer saline 1X PBST) for 5 minutes at 200 rpm, for 3 times. It was then incubated with 200 µl of Blocking Buffer and incubated for 1 hour at room temperature on shaker at 200 rpm. The plate was then washed again with 200 µl of PBST for 5 minutes, three times at room temperature and at 200 rpm. The wells were then coated with 100 µl of Primary antibody (IgM, IgG and IgG_4 – human serum sample) and incubated at room temperature for 1 hour at 200 rpm.

After incubation, the wells were then washed off with 200 µl of PBST for 5 minutes, three times at 200 rpm. The wells were then coated with 100 µl of Secondary antibody (anti-human IgM, IgG and IgG_4) and then incubated at room temperature, for 1 hour at 200 rpm. The plate was then washed again with 200 µl of PBST for 5 minutes, three times at 200 rpm.

After washing, 100 µl of TMB substrate was added to each well, which turns the solution into blue colour and was incubated in a dark room for 15 minutes. Then 100 µl of stock solution was added to each well, turning the blue colour solution into yellow. Then the plate was read in ELISA reader where the concentration at each well was read and recorded

2.2 Optimization of Primary Antibody

2.2.1 Dilution of Primary Antibody

30.0 µl of Primary antibody (human sera samples – ALA +ve & IHA -ve) was dissolved in 750 µl of Phosphate Buffer. Then in a separate ELISA plate, in the first row, 250 µl of antigen solution was added. In the other rows, 125 µl of Phosphate buffer solution was added. Then from the first row, using a multipipetter, 125 µl of antigen solution was taken and put in the second row, mixing it at least 4-5 times and then transferring into the third row again and mixing, going on until the last row, diluting the concentration (1:25, 1:50..... 1:3200) of antigen for each row, which was used in ELISA later on.

2.2.2 ELISA (Enzyme Linked Immunosorbent Assay)

First, 100µl of antigen solution (CSA) was added to each well in an ELISA plate and was incubated overnight at 4°C in a moist chamber.

After overnight incubation, the plate was incubated at room temperature (25°C) for 1 hour on a rotator at 200 rpm (rotation per minute). The plate was then washed with 200 µl of 1X PBST (Twee-20 dissolved in 1X Phosphate buffer saline) for 5 minutes at 200 rpm, for 3 times. It was then incubated with 200 µl of Blocking Buffer and incubated for 1 hour at room temperature on shaker at 200 rpm. The plate was then washed again with 200 µl of PBST for 5 minutes, three times at room temperature and at 200 rpm. The wells were then coated with 100 µl of Primary antibody (IgM, IgG and IgG_4 – human serum sample) and incubated at room temperature for 1 hour at 200 rpm.

After incubation, the wells were then washed off with 200 µl of PBST for 5 minutes, three times at 200 rpm. The wells were then coated with 100 µl of Secondary antibody (anti-

human IgM, IgG and IgG_4) and then incubated at room temperature, for 1 hour at 200 rpm. The plate was then washed again with 200 µl of PBST for 5 minutes, three times at 200 rpm.

After washing, 100 µl of TMB substrate was added to each well, which turns the solution into blue colour and was incubated in a dark room for 15 minutes. Then 100 µl of stock solution was added to each well, turning the blue colour solution into yellow. Then the plate was read in ELISA reader where the concentration at each well is read and recorded to determine the ideal concentration for primary antibody.

2.3 Optimization of Secondary Antibody

2.3.1 Dilution of Secondary Antibody

8.0 µl of Secondary Antibody (Anti IgG, Anti IgM & Anti IgG_4) was dissolved in 2.0 ml of Phosphate Buffer. Then in a separate ELISA plate, in the first row, 250 µl of antigen solution was added. In the other rows, 125 µl of Phosphate buffer solution was added. Then from the first row, using a multipipetter, 125 µl of antigen solution was taken and put in the second row, mixing it at least 4-5 times and then transferring into the third row again and mixing, going on until the last row, diluting the concentration (1:250, 1:500..... 1:32000) of antigen for each row, which was used in ELISA later on.

2.3.2 ELISA (Enzyme Linked Immunosorbent Assay)

First, 100 µl of antigen solution (CSA) was added to each well in an ELISA plate and was incubated overnight at 4°C in a moist chamber.

After overnight incubation, the plate was incubated at 25°C (room temperature) for 1 hour on a rotator at 200 rpm (rotation per minute). The plate was then washed with 200 µl of

1X PBST (Twee-20 dissolved in 1X Phosphate buffer saline) for 5 minutes at 200 rpm, for 3 times. It was then incubated with 200 µl of Blocking Buffer and incubated for 1 hour at room temperature on shaker at 200 rpm. The plate was then washed again with 200 µl of PBST for 5 minutes, three times at room temperature and at 200 rpm. The wells were then coated with 100 µl of Primary antibody (IgM, IgG and IgG_4 – human serum sample) and incubated at room temperature for 1 hour at 200 rpm.

After incubation, the wells were then washed off with 200 µl of PBST for 5 minutes, three times at 200 rpm. The wells were then coated with 100 µl of Secondary antibody (anti-human IgM, IgG and IgG_4) and then incubated at room temperature, for 1 hour at 200 rpm. The plate was then washed again with 200 µl of PBST for 5 minutes, three times at 200 rpm.

After washing, 100 µl of TMB substrate was added to each well, which turns the solution into blue colour and was incubated in a dark room for 15 minutes. Then 100 µl of stock solution was added to each well, turning the blue colour solution into yellow. Then the plate was read in ELISA reader where the concentration at each well was read and recorded to determine the ideal concentration for secondary antibody.