Evaluation of *Entamoeba histolytica* Acetyl Co-A Synthetase Recombinant Protein (rEhACS) for Diagnosis of Acute Amoebic Liver Abscess in Moribund Hamsters

by

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Thesis submitted in partially fulfillment of the requirements for the course GTB 409 Research Project of Bachelor of Health Sciences (Biomedicine)

2013

ACKNOWLEDGEMENT

First of all, I will like to take this opportunity to express my sincere thanks to Dr. Lim Boon Huat, my supervisor for his advices and guidance on this Final Year Research Project. A special thanks to our final year project coordinator, Dr. See Too Wei Cun, who always giving us comments and encouragement.

I also want to say thank Wong Weng Kin and Foo Phiaw Chong, my seniors who guided me on the technical aspects of my research project. They patiently taught me many useful knowledge and skills related to my project, and were always kind and gentle whenever I seek their advice. I wish to acknowledge the financial support that I obtained from the Ministry of Education Long Term Research Grant (LRGS) number 203/PSK/6722002 on Tropical Tuberculosis: Delineating Host-Environment-Pathogen Interactions (TB-Parasite Co-Infection: A Correlation Study).

On a more personal note, I would like to dedicate this thesis to my family and express my deepest gratitude to them for their unwavering love, support and inspiration. I hope that I have made them proud.

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMNS

~	About
%	Percentage
>	More than
^0C	Degree Celsius
µg	Microgram
µL	Microliter
ALA	Amoebic liver abscess
CBB	Coomassie brilliant blue
cm	Centimeter
mm	Millimeter
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 hemagglutination assay
kDa	Kilodalton
K_2HPO_4	Dipotassium phosphate
KH_2PO_4	Monopotassium phosphate
L	Litre
mA	MiliAmpere
h	Hour
min	Minute
mL	Milliliter

mM	Milimolar
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NC	Nitrocellulose
OD	Optical density
TBS	Tris-Buffered Saline
TBST	TBS-Tween 20
PBS	Phosphate Buffered Saline
PBST	PBS-Tween 20
psi	Pound-force per square inch
rEhACS	Recombinant *Entamoeba histolytica* Acetyl Co-A Synthetase
s	Second
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TMB	3,3',5,5'-Tetramethylbenzidine
rpm	Revolutions per minute
LB	Luria Bertani

Penilaian Protein Rekombinan *Entamoeba histolytica* Acetyl Co-A Synthetase (rEhACS) untuk Diagnosis Abses Hati Amoeba Akut pada Hamster Nazak

ABSTRAK

Abses hati ameba (ALA) yang disebabkan oleh jangkitan *Entamoeba histolytica* boleh membawa maut. Di Hospital Universiti Sains Malaysia, diagnosis ALA termasuklah pengesanan antibodi dengan menggunakan kit komersial yang berasaskan antigen larut mentah (CSA) *E. histolytica.* CSA terdiri daripada pelbagai protein *E. histolytica* yang mana ciri dan jisim setiap protein tidak diketahui dengan tepat. Kajian ini bertujuan untuk menilai potensi diagnostik protein rekombinan *E. histolytica* sintetase asetil Co-A (rEhACS) dengan menggunakan sampel serum daripada hamster yang nazak akibat jangkitan ALA. rEhACS telah dihasilkan dalam *E. coli* BL21 AI dan ditulenkan dengan menggunakan kolum resin Ni-NTA dalam keadaan yang optimum. Ketulenan protein rekombinan dinilai menerusi gel SDS-PAGE yang diwarnakan dengan *Coomassie brilliant blue*. Selain daripada jalur ~77 kDa, tiada jalur lain diperhatikan pada gel tersebut. Spesifisiti dan sensitiviti rEhACS tulen dinilai menerusi asai pemedapan Western dan asai imunojerapan berpaut enzim (ELISA) dengan menggunakan 31 sampel serum hamster yang ALA positif dan lima sampel serum daripada hamster sihat. Analisis pemedapan Western menunjukkan rEhACS adalah 58.07% sensitif dan 100% spesifik; manakala rEhACS yang diguna dalam format ELISA adalah 100% spesifik dan sensitif. Kesimpulannya, protein rekombinan EhACS ~77 kDa telah dibuktikan penting untuk diagnosis ALA dalam model haiwan dan potensinya untuk diagnosis ALA pada manusia akan dinilai selanjutnya dalam kajian masa depan.

Evaluation of *Entamoeba histolytica* Acetyl Co-A Synthetase Recombinant Protein (rEhACS) for Diagnosis of Acute Amoebic Liver Abscess in Moribund Hamsters

ABSTRACT

Amoebic liver abscess (ALA) is a potentially fatal infection caused by *Entamoeba histolytica*. At Hospital Universiti Sains Malaysia, diagnosis of ALA includes detection of antibodies using a commercial kit based on crude soluble antigens (CSA) of *E. histolytica*. CSA consisted of a cocktail of *E. histolytica* proteins that are not well-defined in terms of the characteristics of the proteins and their masses. This study aimed to evaluate the diagnostic potential of a well-defined recombinant protein of *E. histolytica* acetyl-Co-A synthetase (rEhACS) using serum samples from hamsters with acute fatal ALA. The rEhACS was expressed in *E. coli* BL21 AI and purified using Ni-NTA resin column under optimized parameters. Purity of the protein was evaluated based on observation of the ~77 kDa on SDS-PAGE gel stained with Coomassie brilliant blue. No unspecific band was observed in the gel. The specificity and sensitivity of the purified protein was evaluated via Western blot and enzyme-linked immunosorbent assay (ELISA) using 31 ALA-positive and 5 healthy hamster serum samples. Western blot analysis showed that the sensitivity and specificity of rEhACS were 58.07% and 100% respectively; while the sensitivity and specificity of the recombinant EhACS used in an ELISA format were both 100%. In conclusion, the ~77 kDa recombinant EhACS protein was shown to be important for diagnosis of acute fatal ALA in the animal model and its diagnostic potential in human ALA will be evaluated in future study.

CHAPTER ONE

INTRODUCTION

1.1 Introduction to *Entamoeba histolytica*

Entamoeba histolytica is an intestinal protozoan parasite and the causative agent of invasive amebiasis. Prior to the introduction of molecular diagnostics, it was reported that about one-tenth of the world population was infected by *E. histolytica* (Walsh, 1986) resulting in up to 100,000 deaths worldwide each year (Anon, 1997; Petri *et al.*, 2000; Haque *et al.*, 2003a). Although the deaths could be due to invasion of *E. histolytica*, the prevalence data of *E. histolytica* infection was an overestimation because the pathogenic *E. histolytica* was not distinguishable from the non-pathogen *E. dispar* via microscopy (Diamond and Clark, 1993). Interestingly, recent studies reported that *E. moshkovskii*, another morphologically identical species, was also common in some endemic areas of *E. histolytica* (Ali *et al.*, 2003; Parija and Khairnar, 2005; Khairnar *et al.*, 2007; Beck *et al.*, 2008; Fotedar *et al.*, 2008). It is highly possible that, *E.dispar and E. moshkovskii* were considered as *E. histolytica* in the old epidemiological data. It remains the first priority for researchers to obtain species specific prevalence data in order to fill in the current knowledge gaps in many geographical regions.

Researchers have noticed that not all *E. histolytica* infection could lead to disease progression (Walker and Sellards, 1913). It was suggested that one in four persons infected with *E. histolytica* would suffer from disease onset (Gathiram and Jackson, 1985; Blessmann *et al.*, 2003; Haque *et al.*, 2006). Nevertheless, *E. histolytica* remains a significant cause of morbidity and mortality in developing

countries (Haque *et al.*, 2003a; Stanley, 2003). For example, the annual incidence of amebic dysentery in pre-school children is 2.2% compared to 5.3% for *Shigella* dysentery in Bangladesh, a country where one in every 30 children dies of diarrheoa or dysentery before reaching his or her fifth birthday (Haque *et al.*, 2003b). Similarly, the annual incidence of amebic liver abscess (ALA) averaged 21 cases per 100,000 inhabitants in Hue City, Vietnam (Blessmann *et al.*, 2002).

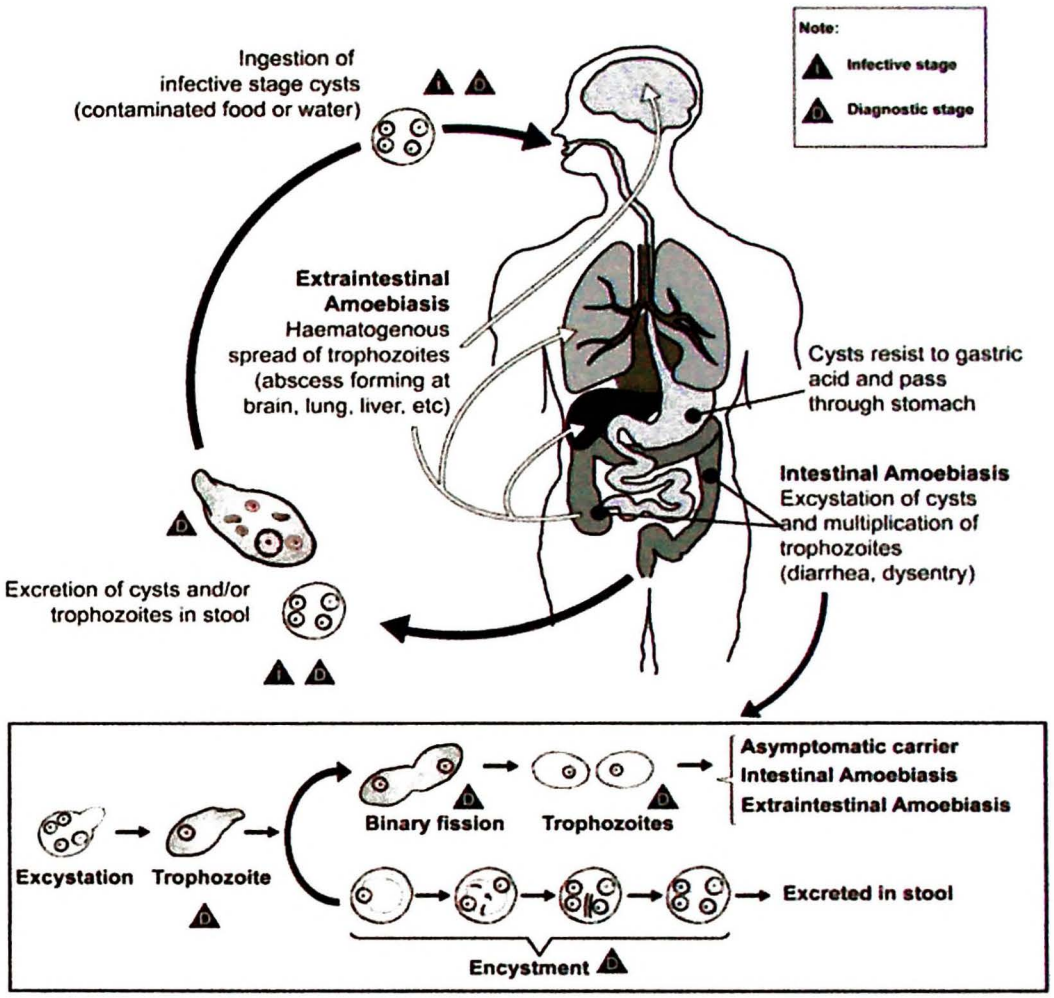
Only a small percentage of persons infected with *E. histolytica* showed onset of clinical signs and symptoms like intestinal amoebiasis e.g. diarrheoa or dysentery, or extraintestinal amoebiasis e.g. ALA. However, the underlying fundamental mechanism that caused the onset of intestinal or extraintestinal amoebiasis was still unresolved. A study by Ali *et al.* (2007) suggested that the different types of parasite genotypes may have association with the disease outcome of infection by *E. histolytica*. Research works is still on going to look for specific genes that are associated with the disease outcome of an *E. histolytica* infection in this post-genomic era.

1.2 Life Cycle of *E. histolytica*

The life cycle of *E. histolytica* consists of two main stages i.e. the trophozoite and the cyst form (Sehgal *et al.*, 1996). Trophozoite is the motile and active multiplying form. On the other hand, cyst is the dormant and highly resistant form. It is resistant to pH and environmental temperature changes. The infective stage cysts enter human body through ingestion of contaminated food or water with faecal material. Cysts survive from the stomach acid passed into the intestinal region. Each cyst undergoes excystation and transform into eight trophozoites. The motile

trophozoites will migrate to the colon region in the intestine and start to actively multiply in that region. In most of the person infected with *E. histolytica*, trophozoites live commensally together with the other intestinal flora in the colon. Eventually, trophozoites may initiate their invasion towards the intestinal mucosal layer. This will lead to the onset of disease symptoms like diarrhea and dysentery. In some serious cases, trophozoites may break into the blood circulation and spread haematogenously throughout the body. This will lead to extraintestinal amoebiasis in location like liver, brain and lungs. The most common clinical outcome was amoebic liver abscess and it is life-threatening.

As the life cycle progress, the trophozoites will undergo encystment and produce quadrinucleate cysts. Both forms of *E. histolytica* will be excreted along with the faeces. However, only the highly resistant cysts can be remained viable for prolonged period of time outside the host. The trophozoites could only survive for few hours outside the host. *E. histolytica* cysts serve as the infective agent while the trophozoites were responsible for the disease progressing. The 'carrier' or asymptomatic human host was responsible for continuously passing out the infective stage cysts to the environment (Figure 1.1).



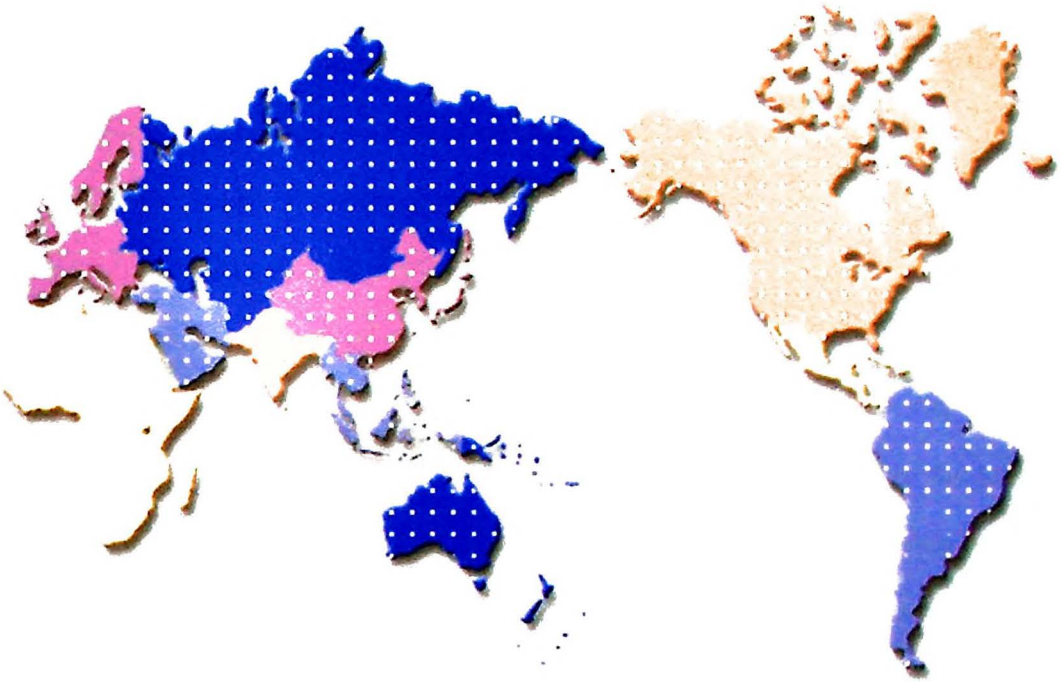
(Image was adopted and modified from http://www.dpd.cdc.gov/dpdx/html/amebiasis.htm)

Figure 1.1 Life cycle of *E. histolytica*

1.3 Epidemiology of Amoebiasis

Amoebiasis was reported worldwide especially in the tropical and temperate zones (Figure 1.2). Epidemiological studies carried out over the past few years were facing problem with the finding of a nonpathogenic and also morphologically identical *Entamoeba* species which is *E. dispar*. Nowadays, advances in molecular diagnostic led to the identification of another morphologically similar amoeba, namely *E. moshkovskii* (Fotedar *et al.*, 2007b). Nonetheless, advanced screening methods employed in recent studies still reported a higher prevalence of *E. histolytica* in study populations (Ramos *et al.*, 2005a; Ramos *et al.*, 2005b). Recently, a study found that two-thirds of patients in mental institutions located in Manila, Philippines are positive for *E. histolytica* (Rivera *et al.*, 2006). Currently, molecular and epidemiological studies are being employed to genotype of *E. histolytica* strains based on polymorphisms in the serine-rich *E. histolytica* protein (SREHP) locus, with emphasis on studying the geographic diversity of the virulent strains (Ayeh-Kumi *et al.*, 2001; Haghighi *et al.*, 2002; Haghighi *et al.*, 2003).

Epidemiological studies also mentioned the importance of host factors in *E. histolytica* infection especially when the re-infection rates in a study population have been found to be two point seven times higher when compared with the primary infection rates 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 currently, protection against the *E. histolytica* infection is thought to be genetically linked to HLA class II-restricted immune response.



(Image Source: http://atlas.or.kr/atlas/alphabet_view.php?my_codeName=*Entamoeba%20histolytica*)

Figure 1.2 Global distribution of amoebiasis. Amoebiasis is found worldwide especially in tropical and temperate zone. A dot indicates an area where amoebiasis is present.

1.4 Pathogenesis of *E. histolytica*

Current technology could not allow researcher to perform in vivo study of the pathogenesis of amoebiasis in a human model. Both in vivo and in vitro models were reported for the study of pathogenesis in amoebiasis. In vitro models involved the culture of the trophozoites in cancer cell lines. Virulence trophozoites would lyse most of the cancer cells within 48 hours of co-culture. However, researchers could not simulate the abscess development in this model. The alternative way was to simulate the disease in animal models. Several species have been used as the animal models for in vivo study of various aspects in pathogenesis of amoebiasis such as hamsters and gerbils (Meerovitch *et al.*, 1988). They were also the commonly used animal models for the study of amoebic liver abscess. In experimentally induced amoebic liver abscess in these animal models, the virulence trophozoites were injected into the liver through hepatic portal vein and the lesion would developed in five to seven days post inoculation.

Figure 1.3 shows the schematic representation of *E. histolytica* pathogenesis (Wiser, 2011). Cysts will undergo excystation and develop into trophozoites. The trophozoites will colonize the colon and interact with the normal flora in the colon and undergo further changes. A study by Wittner and Rosenbaum (1970) reported that direct interaction between trophozoites and viable bacteria was pertinent for the virulence of the trophozoites. In an in vivo study by Bracha and Mirelman (1984), trophozoites' virulence was increased after exposure to bacteria for 30 min. Nevertheless, bacteria interaction was not an absolute necessity for invasion of trophozoites. Interaction with the cell surface would change the cell surface constitutions of trophozoites and lead to alteration in its characteristics. Normal flora

presents in the guts would provide some nutrition requirements and a low oxygen tension environment which favor the colonization of trophozoites.

The major *E. histolytica* virulence factors involved in pathogenesis are shown in Table 1.1 (Lejeune *et al.*, 2009). Invasions of virulence trophozoites started with the adherence to mucosal layer of the intestine. In this case, the *E. histolytica* surface membrane protein, Gal/GalNAc lectin plays the main role in the adherence process. This surface membrane protein has high binding affinity to O-linked protein, galactose (Gal) and N-acetyl-D-galactosamine (NAcGal) that is present in the epithelial cell. Sehgal *et al.* (1996) has shown that pretreatment of trophozoites with GalNAc would inhibit the adherence of the trophozoites to the mucosal layer.

After that, lysis of the cells is prior to mucosal invasion by this parasite. Amoebic microfilament function such as Ca^{2+} flux and phospholipase A are the requirement for cytolysis of the target cell. Several of amoebic pore-forming proteins had been described by Dodson and Petri (1994). One of the pore-forming proteins named as the 30 kDa amoebic protein was shown to lysed erythrocytes and insert into and create pores in lipid bilayers. Another protein, 14 kDa pore-forming protein is an ion-channel forming protein. Neutrophils and leucocytes also play important role in tissue destruction (Tanyuksel and Petri, 2003; Leippe and Herbst, 2004). They release toxic product to lyse the cells. Upon invasion of the trophozoites into the bloodstream, they would be spread to many vital organs in human body. It may cause abscess to form in the liver, brain and lungs. Trophozoites isolated from stool of invasive patient may have higher rate of erythrophagocytosis activities and was suggested to be associated to the virulence by Orozco *et al.* (1983).

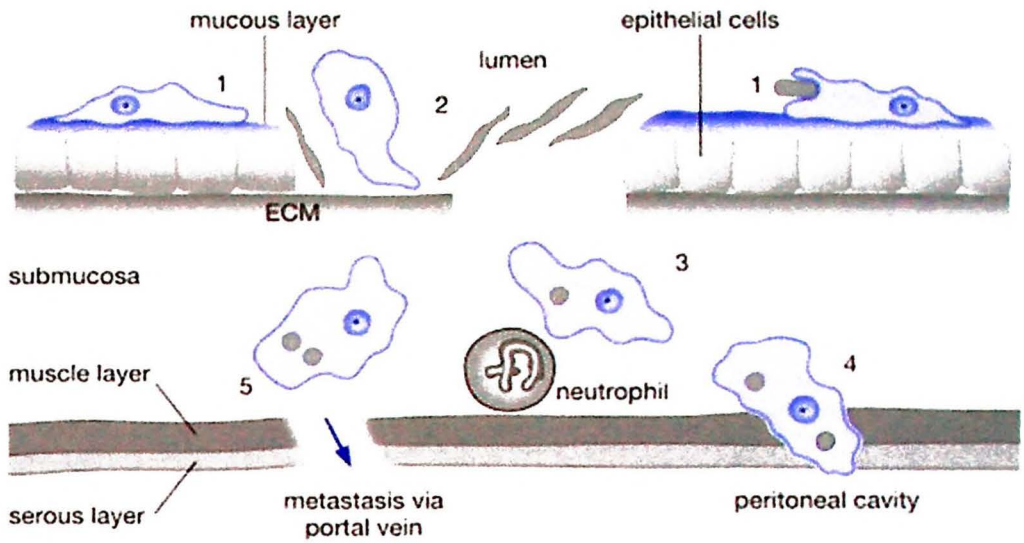


Figure 1.3 Schematic representation of *E. histolytica* pathogenesis (Wiser, 2011)

Table 1.1 The major *E. histolytica* virulence factors involved in pathogenesis (Lejeune *et al.*, 2009)

***E. histolytica* molecule**	**Role in pathogenesis**
EhCP	• Cleaves MUC2 polymer specifically at the cysteine-rich C-terminal domains and depolymerizes mucins. • Cleaves immunoglobulins, complements, as well as cytokines such as IL-18. • Signaling role in the development of ALA.
Gal-lectin	• Surface adhesion molecule in parasite colonization. • Mediates apoptotic death of epithelial cells that the trophozoites contact.
LPPG	• Parasite surface molecule utilized for epithelial adhesion and cytotoxicity.
EhSTIRP	• Parasite surface molecule utilized for epithelial adhesion and cytotoxicity.
Perioxireoxin	• Neutralize ROS and NO, which are released from activated macrophages.
Ehserp	• Forms a complex with cathepsinG that are released from neutrophils and neutralizes them.
Eh arginase	• Converts L-arginine, the precursor of NO, into L-ornithine and thus hinders NO production in the macrophage.
β1 integrin-like receptors	• Interact with fibronectin and laminin of endothelial cells as well as extracellular matrix protein, and activate trophozoites that enter systemic circulation.
Amoebapore	• Pore-forming peptide that plays a major role in ALA.
KERP1	• Specifically upregulated on parasite surface during ALA, but the role in pathogenesis is not clearly known.

ALA: Amoebic liver abscess; Eh: *Entamoeba histolytica*; EhCP: *E. histolytica* cysteine proteinase; Ehserp: *E. histolytica* serine proteinase; EhSTIRP: Serine threonine isoleucine-rich protein; KERP1: Lysine and glutamic acid-rich protein-1; MUC2: Secretory mucin; LPPG: Lipophosphopeptidoglycan; NO: Nitric oxide; ROS: Reactive oxygen species.

1.5 Diagnosis of *E. histolytica* Infection

1.5.1 Current Diagnosis

According to Fotedar *et al.* (2007a), microscopy, culture, antigen detection, serology test and polymerase chain reaction are currently used in the laboratory for diagnosis of amebiasis. Microscopic stool examination for trophozoites is only 33-50% sensitive from a stool sample in amebic colitis. Usually the results for stool examination findings in patients with amebic liver abscess are usually negative. The World Health Organization (WHO) suggests that intestinal amebiasis be diagnosed with an *E. histolytica*-specific test, thus the classic stool ova and parasite examination is still rendering. Until today, most of the laboratory still refers to microscopic test to confirm the result of amoebiasis from other test.

Variations of serologic assays are now available for the diagnosis of amebiasis. The most commonly used assay to measure the presence of serum anti-lectin antibodies (IgG) is ELISA. The sensitivity and specificity is 97.9% and 94.8%, respectively, for the detection of antibodies to *E histolytica* in patients with ALA. False-negative results may occur within the first 7-10 days post-infection by this parasite. Immunofluorescent assay (IFA) is also used as a test for ALA because it is rapid, reliable, and reproducible. The sensitivity and specificity of IFA was shown to be 93.6% and 96.7% respectively. Indirect hemagglutination (IHA) is less sensitive than ELISA. Immunoelectrophoresis, counter-immunoelectrophoresis (CIE), and immunodiffusion tests both can be used for detecting ALA. They use the precipitation property of antigen-antibody complexes in agar. CIE is consumed a lot of time but its sensitivity is 100% to detect invasive amebiasis. Complement fixation

(CF) is less sensitive. The seropositivity prevalence is very high in endemic areas, limiting antibody-based testing for diagnosing currently active disease, since the antibodies can persist for years after infection (Fotedar *et al.*, 2007a).

E. histolytica can be found in various types of clinical specimens, such as feces, tissues, and liver abscess aspirates. PCR methods targeting different genes, for example, a small-subunit rRNA gene (18S rDNA), 30-kDa antigen gene, serine-rich protein gene, chitinase gene, hemolysin gene, and extrachromosomal circular DNA. These techniques can be used for the detection and differentiation of *E. histolytica*, *E. dispar*, and also *E. moshkovskii*. Sensitivities can vary according to the sampling and the specific target gene used. PCR-based tests have been strongly endorsed by the WHO (Fotedar *et al.*, 2007a).

1.5.2 Problem in Diagnosis of *E. histolytica* Infection

Currently, diagnosis of *E. histolytica* infection is based on the direct microscopic identification of the parasite. This technique is not sensitive and cannot differentiate between pathogenic *E. histolytica* from the noninvasive *E. dispar*. According to WHO, *E. histolytica/E. dispar* should be reported if diagnosed by light microscopy alone. Serology is very useful in the diagnosis of extraintestinal amebiasis (Wiser, 2011). Approximately 70-80% of patients with acute invasive colitis or liver abscesses have serum antibodies against *E. histolytica*. However, distinguishing past and current infections might be difficult because these antibodies can persist for years especially in endemic areas. Non-invasive imaging techniques such as ultrasound, computerized tomography, and magnetic resonance imaging can be used to detect liver abscesses, but, this method is rarely done in the laboratory and

only carried out for some selected cases such as serology and imaging and not available for therapeutic purposes. The aspirate usually looks like a thick reddish brown liquid that rarely contains trophozoites. Trophozoites are most likely to be found at the abscess wall and not in the necrotic debris at the abscess center. Therefore, an improved method for detecting *E. histolytica* is still needed. An expert consultation on amoebiasis in Mexico City stressed that there is a need to improve the methods for the specific diagnosis of *E. histolytica* infections based on appropriate technologies for use in developing world (WHO, 1997).

1.6 Rationale of Study

1.6.1 Previous Work on This Project

Previously, *E. histolytica* was cultured axenically and then used to infect the liver of hamster. The infected liver was harvested for the trophozoites. The trophozoite antigens were prepared by using lysis buffer and sonication. Since *E. histolytica* produce a lot of proteases, protease inhibitor was added to inhibit their activities. Based on 2-D Western blot analysis, against the ALA serum samples, the antigenic protein band was sent for sequencing, where ACS was confirmed.

Subsequently, molecular cloning was performed. First, the genomic DNA of the trophozoites was extracted and specific primers were designed. PCR amplification was performed by using both *Taq* polymerase and *Pfu* polymerase. The ACS gene was then cloned into the vector pET-14b and sent for sequencing to confirm the gene insertion. Then, the recombinant ACS (rACS) was cloned into the expression bacteria, *Escherichia coli* BL21 (AI) and stored in glycerol at -80 °C. The current study is to evaluate the specificity and sensitivity of this recombinant protein (rEhACS) against ALA hamster antibodies.

1.6.2 Acetyl CoA Synthetase (ACS) as a potential antigen for serodiagnosis

Reeves *et al* (1977) reported a study on the core metabolism of the amitochondriate eukaryote *E. histolytica.* They detected an enzyme that produced acetate, a major metabolic end product of this organism. This enzyme, ACS (ADP-forming), which is also called acetate thiokinase, catalyzes the reaction: acetyl CoA + ADP + phosphate $\rightleftarrows$ acetate + CoA + ATP (Figure 1.4). This enzyme, remained

an oddity, was found only in *E. histolytica* and *Giardia lamblia* with Type 1 amitochondriate core metabolism (Müller *et al.*, 1998; Martin and Muller, 1998). It should not be confused with ACS (AMP-forming), an unrelated enzyme present in many bacteria and eukaryotes. Identification and sequence analysis of the *G. lamblia* ACS resulted in the identification of the homologous *E. histolytica* ACS (Field *et al.*, 2000), and the identification of an ACS gene homolog in the malaria parasite, *Plasmodium falciparum* (Sanchez *et al.*, 2000).

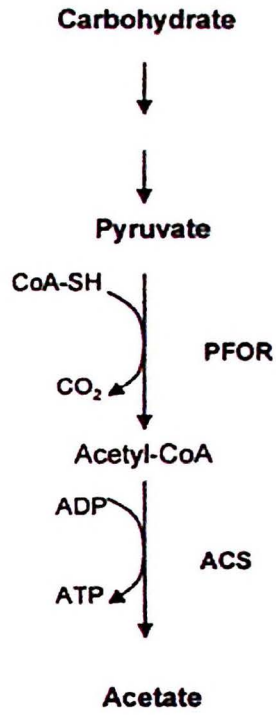


Figure 1.4 Acyl-CoA synthetases in α-keto acid catabolism for *E. histolytica* and *G. lamblia* (Sanchez *et al.*, 1999).

ACS-ADP enzymes are either heterotetrameric proteins that consist of two separate α and β subunits (α2, β2) or homodimers that consist of two subunits representing a fusion of the respective α and β subunits $(\alpha\beta)_2$. The α and β subunits of ACS-ADP forming are homologous to the α and β subunit of succinyl-CoA synthetases and together these enzymes belong to the super family of nucleoside diphosphate-forming (NDP-forming) acyl-CoA synthetases (Field *et al.*, 2000; Sanchez *et al.*, 2000; Musfeldt and Schonheit, 2002; Brasen *et al.*, 2008).

1.7 Objectives of the Study

1) To optimize the over-expression of rEhACS in *E. coli* BL21 AI.
2) To optimize the purification steps of the expressed rEhACS.
3) To optimize ELISA protocols based on rEhACS.
4) To evaluate the potential of rEhACS in diagnosis of ALA of hamster by using Western blot assay and ELISA.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Optimization of Protein Over-expression

In this study, recombinant EhACS was over-expressed in *E. coli* BL21 AI containing pET-14b/ACS plasmid. The parameters, i.e. durations and temperatures for the protein over-expression and solubility were optimized.

2.1.1 Protein Over-expression

E. coli BL21 AI containing pET-14b/ACS plasmid was transferred from glycerol stock into 5 mL Luria-Bertani (LB) culture medium (pH 7.0) supplemented with ampicillin (100 μg/mL). The tube containing the cell suspension was shook at 200 rpm for overnight at 37 °C. On the following day, 1 mL of overnight culture was transferred into a conical flask containing 55 mL of LB medium supplemented with ampicillin (100 μg/mL) and shook for 2 hours at 200 rpm, 37°C, until the optical density (OD_{600}) reached 0.6 – 0.8. One mL of the pre-induced culture was transferred into a 1.5 mL microcentrifuge tube; centrifuged at 10, 000 × *g* for 5 min, 4 °C; and the cell pellet was kept at -80 °C for later analysis. Next, 500 μL of 20% L-arabinose was added into the cell suspension. Subsequently, 5 mL of the cell suspension was aliquoted into several 15 mL centrifuge tubes and shook at 200 rpm with different durations and temperature as stated Table 2.1.

Table 2.1 Parameters for optimization of protein over-expression

Temperature	22 °C	27 °C	32 °C	37 °C
Duration	1 h	1 h	1 h	1 h
	3 h	3 h	3 h	3 h
	6 h	6 h	6 h	6 h
	O/N	O/N	O/N	O/N

Upon completion of each culture condition, 1 mL of cell suspension from each tube was pipette out for OD_{600} measurement and then put back into the respective tube. The 5 mL cell suspensions were then centrifuged for 20 min at 4000 rpm, 4 °C. The supernatants were discarded and the cell pellets were resuspended with 500 µL of Lysis Buffer (38.72 mM Tris-HCl, 299.45 mM NaCl, pH 7.5) supplemented with protease inhibitor (Roche, USA). The cell suspension was sonicated on ice for at least 3 X 1 min cycles at 10 % amplitude with 0.5 s pulse-on and 0.5 s pulse-off. Additional cycle was performed until the cell suspension become clear. The cell lysates were centrifuged for 10 min at 10, 000 × *g*, and 4 °C. The supernatants and the pellets were kept separately at -80 °C for later analysis.

2.1.2 Analysis of Protein Over-expression *via* SDS-PAGE

Expressed protein was separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). This technique was performed as described by Laemmli in 1970 with some modifications. Stacking and resolving gels were casted based on the technical bulletin of Bio-Rad Mini Protean III Electrophoresis Cell (Bio-Rad, USA). The ratio of buffers and reagent for the gel casting were listed in Table 2.2. In brief, small gel [8 cm (W) X 7.3 cm (H) X 0.75 mm (T) made of 9% resolving gel and 3% stacking gel] was casted and assembled according to the technical bulletin prior to the running of SDS-PAGE. The protein samples with different protein amounts were mixed with 2X sample buffer in the ratio of 1:1 and boiled for 5 min. The boiled samples were then centrifuged at 10, 000 X g for 30 seconds and the supernatant from each sample was loaded into the wells of stacking gel. The electrophoresis was run at a constant current with 25 mA per gel until the Bromophenol blue dye was ~0.5 cm from the bottom of the glass plate which took about 1 hr. Upon completion of the electrophoresis, the resolving gel was carefully removed from the glass plate which is apart with stacking gel and was stained in CBB (Bio Basic, Canada).

Table 2.2 Gel casting ingredient for SDS-PAGE

Ingredient	Resolving Gel (9 %)	Stacking Gel (3 %)
Resolving Buffer, pH 8.8	2.5 mL	-
Stacking Buffer, pH 6.8	-	1.25 mL
Acrylamide, 30 %	3 mL	0.5 mL
SDS, 10 %	100 µL	50 µL
APS, 10 %	100 µL	50 µL
TEMED	10 µL	5 µL
dH2O	4.29 mL	3.15 mL
Total	**10.00 mL**	**5.005 mL**

2.2 Optimization of Protein Purification

2.2.1 Imidazole Concentration

In this section, imidazole concentration in washing buffer during the washing of the Ni-NTA resin was optimized while the number of washing step was fixed at 5 times. A spike of *E. coli* BL21 AI from glycerol stock was put in 5 mL of LB medium supplemented with ampicillin (100 µg/mL) and shook overnight at 200 rpm, 37 °C. One mL of the overnight culture was transferred into a fresh 100 mL LB medium supplemented with ampicillin (100 µg/mL) and shook at 200 rpm, 37 °C for 2 hours until OD_{600} reached 0.6 – 0.8. Upon reaching OD_{600} 0.6 – 0.8, 500 µL of 20% L-arabinose was added into the culture. The culture was incubated in a shaker at the optimised condition (as determined in Section 2.1) at 200 rpm, 32 °C. The overnight culture was then transferred into the 50 mL centrifuge tube and centrifuged at 4000 rpm, 4 °C for 20 min. The supernatant was discarded while the cell pellet was resuspended with 5 mL of Lysis buffer added with protease inhibitor. The cell suspension was sonicated on ice for at least 3 x 1 min cycles at 10% amplitude with 0.5 s pulse-on and 0.5 s pulse-off. Additional cycle was performed until the cell suspension become clear. The cell lysate was distributed evenly into new 1.5 mL microcentrifuge tubes and centrifuged for 10 min at 10, 000 × *g*, and 4 °C. The pellet and supernatant were kept separated at -80 °C until used.

His-Tag recombinant EhACS was purified using HisPur™ Ni-NTA Purification Kit (ThermoScientific, USA) according to the manufacturer manual. First, the resin was washed with lysis buffer for three times. Two hundred µL of lysis buffer was added into a purification column containing 200 µL of resin bed. Then the

purification column was centrifuged at 700 × *g*, 4 °C for 2 min. The flow through was discarded. These steps were repeated for three times. Next, the washed resin was mixed with the cell lysate supernatant for the resin to capture the rEhACS protein. Four hundred µL of the supernatant was mixed with the washed resin and incubated overnight in cold room (4 °C) with continuous rotation.

The resin with captured rEhACS protein was washed with washing buffer containing five different concentrations of imidazole i.e. 20 mM, 40 mM, 60 mM, 80 mM, and 100 mM. The overnight incubated columns centrifuged at 700 × g, 4 °C for 2 min to remove the cell lysate. The resin in the columns was then added with 200 µL of washing buffer with different concentration of imidazole, respectively. The washing steps were repeated five times. Upon completion of the resin washing, 200 µL of elution buffer (38.44 mM Tris-HCl, 299.80 mM NaCl, pH 7.5) was added into each column and then centrifuged for 2 min at 700 × *g*, 4 °C. The eluted protein was analysed using 9% SDS-PAGE to check for the protein purity.