Evaluation of *Entamoeba histolytica* Acetyl Co-A Synthetase Recombinant Protein (r*Eh*ACS) for Serodiagnosis of Acute Amoebic Liver Abscess in Humans

by

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DEDICATION

This dissertation is dedicated to my loving parents

Md Sith Buang and Salbiah Saad

for their unconditional love, support and inspiration.

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LIST OF SYMBOLS

~	About
^{o}C	Degree Celsius
x *g*	Gravity
>	More than
%	Percentage

LIST OF ABBREVIATIONS

cm	Centimeter
dH_2O	Distilled water
et al.	*et alii* – 'and others'
G	Gram
h	Hour
Ig	Immunoglobulin
kDa	Kilodalton
L	Litre
µg	Microgram
µL	Microliter
mA	MiliAmpere
mM	Milimolar
mL	Milliliter
mm	Millimeter
min	Minute
rpm	Revolutions per minute
s	Second
NaCl	Sodium chloride
NaOH	Sodium hydroxide

LIST OF ACRONYMNS

TMB	3,3',5,5'-tetramethylbenzidine
ALA	Amoebic liver abscess
CBB	Coomassie brilliant blue
CSA	Crude soluble antigen
ELISA	Enzyme linked immunosorbent assay
IHA	Indirect hemagglutination assay
LB	Luria Bertani
NC	Nitrocellulose
OD	Optical density
PBS	Phosphate buffered saline
PBST	PBS-tween 20
r*Eh*ACS	Recombinant *Entamoeba histolytica* Acetyl Co-A synthetase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
TBST	TBS-tween 20

Penilaian Protein Rekombinan *Entamoeba histolytica* Acetyl Co-A Synthetase (r*Eh*ACS) untuk Serodiagnosis Abses Hati Amoeba Akut pada Manusia

ABSTRAK

Abses hati ameba (ALA) disebabkan oleh *Entamoeba histolytica* merupakan manifestasi jangkitan ameba luar usus yang berpotensi membawa maut. Di Hospital Universiti Sains Malaysia (HUSM), terdapat pelbagai kaedah diagnosis ALA termasuk pengesanan antibodi dengan menggunakan kit komersial yang berasaskan antigen mentah larut (CSA) *E. histolytica*. CSA terdiri daripada pelbagai protein *E. histolytica* dengan komposisi ciri dan jisim setiap protein tidak diketahui dengan tepat. Kajian ini bertujuan untuk menilai potensi diagnostik protein rekombinan *E. histolytica* sintetase asetil Co-A (r*Eh*ACS) dengan menggunakan sampel serum daripada pesakit di wad HUSM yang disahkan positif ALA berdasarkan simptom klinikal, imbasan radiologi dan ujian serologi positif menggunakan kit komersial haemagglutinasi tidak langsung, IHA (Cellognost® Amebiasis Kit, Dade Behring Marburg GmbH, Jerman). r*Eh*ACS telah dihasilkan dalam *E. coli* BL21 AI dan ditulenkan dengan menggunakan kolum resin Ni-NTA dalam keadaan yang optimum. Protein rekombinan tulen yang mempunyai berat molekul ~77 kDa dilihat menerusi gel SDS-PAGE yang diwarnakan dengan *Coomassie brilliant blue*. Spesifisiti dan sensitiviti r*Eh*ACS tulen dinilai menerusi asai imunojerapan berpaut enzim (ELISA) dengan menggunakan 30 sampel serum pesakit yang disahkan ALA positif dan 30 sampel serum ALA negatif berdasarkan ujian IHA daripada pesakit yang dijangkiti patogen lain selain *E. histolytica*. Sensitiviti dan spesifisiti bagi r*Eh*ACS/total anti-human IgG-ELISA adalah 50% dan 96.67%. Manakala, bagi r*Eh*ACS/anti-human IgG_4-ELISA adalah 36.67% dan 96.67%. Kesimpulannya, kajian ini menunjukkan bahawa r*Eh*ACS adalah sangat

spesifik untuk serodiagnosis ALA. Menariknya, 50% (IgG-ELISA) atau 36.6% (IgG_4-ELISA) dari sampel positif yang digunakan adalah berkemungkinan diperolehi daripada pesakit yang menghidapi ALA akut. Kajian selanjutnya perlu memfokuskan pada mengenalpasti kes ALA akut baru dengan menggunakan antigen r*Eh*ACS diikuti dengan menjalankan pemeriksaan ALA yang menyeluruh untuk menentusahkan jangkitan itu.

Evaluation of *Entamoeba histolytica* Acetyl Co-A Synthetase Recombinant Protein (r*Eh*ACS) for Serodiagnosis of Acute Amoebic Liver Abscess in Humans

ABSTRACT

Amoebic liver abscess (ALA) caused by *Entamoeba histolytica* is the most common and potentially fatal extra-intestinal manifestation of amoebiasis. At Hospital Universiti Sains Malaysia (HUSM), diagnosis of ALA includes detection of antibodies using a commercial kit based on crude soluble antigens (CSA) of *E. histolytica*. CSA consist 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 was done to evaluate the diagnostic potential of a well-defined recombinant protein of *E. histolytica* acetyl-Co-A synthetase (r*Eh*ACS) using serum samples from patients warded in HUSM who were diagnosed with ALA based on clinical symptoms, ultrasound imaging, and positive serology with commercial indirect haemagglutination kit, IHA (Cellognost® Amebiasis Kit, Dade Behring Marburg GmbH, Germany). The r*Eh*ACS was expressed in *E. coli* BL21 AI and purified using Ni-NTA resin column under optimized parameters. Presence of the purified *rEh*ACS protein was ascertained based on observation of the prominently expressed ~77 kDa on SDS-PAGE gel stained with Coomassie brilliant blue. The specificity and sensitivity of the purified protein were evaluated via enzyme-linked immunosorbent assay (ELISA) using 30 ALA-positive serum samples and 30 ALA-negative serum samples with IHA seronegative obtained from patients infected with pathogens other than *E. histolytica*. The sensitivity and specificity for r*Eh*ACS/total anti-human IgG-ELISA were 50% and 96.67% respectively. Meanwhile, for r*Eh*ACS/anti-human IgG_4-ELISA were 36.67% and 96.67% respectively. In conclusion, this study demonstrated that r*Eh*ACS was highly specific for serodiagnosis

of ALA. Interestingly, 50% (IgG-ELISA) or 36.6% (IgG_4-ELISA) of the positive serum samples were probably obtained from patients with acute ALA. Future studies should focus on identifying new acute ALA cases based on r*Eh*ACS antigen followed by performing a battery of ALA examinations such as ultrasonography and molecular detection of biopsied liver abscess samples to confirm the infection.

CHAPTER 1

INTRODUCTION

Amoebic liver abscess (ALA) is the most common fatal manifestation caused by *Entamoeba histolytica* through the hematogenous spread of invasive trophozoites from the colon via hepatic portal vein. The most common non-specific clinical symptoms of ALA include fever and abdominal pain. Tender hepatomegaly is also commonly associated with ALA which is detected on abdominal examination.

1.1 Life cycle of *E. histolytica*

E. histolytica is a pathogenic protozoon which is transmitted through oral faecal route and human can acquire infection by accidental ingestion of the infective cyst of *E. histolytica* in the faecal-contaminated food or water. *E. histolytica* is responsible for a disease called amoebiasis. The infection occurs usually in the large intestine and causes internal inflammation. The parasite has an interchangeable two stage life cycle consisting of an infective cyst form and a motile pathogenic trophozoite form (Das & Ganguly, 2014).

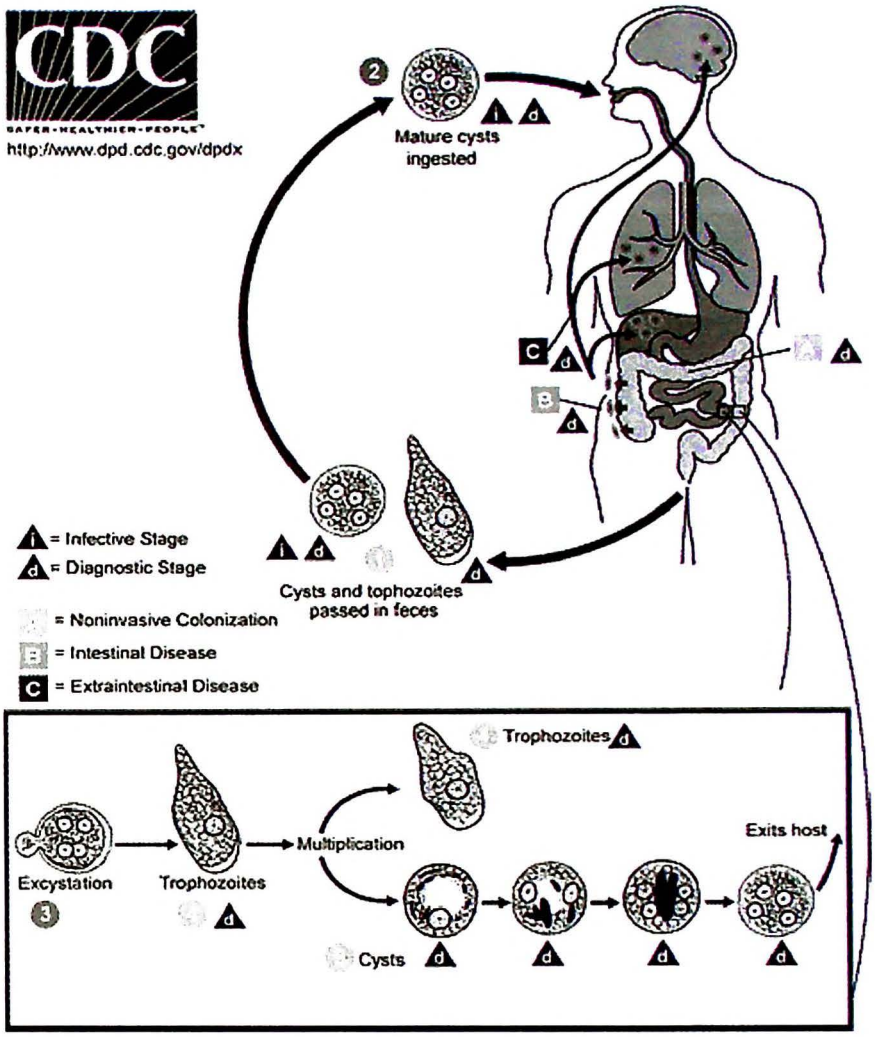
Inside the human body, the cysts transform into trophozoites through a process called excystation in the small intestine which will then reside in the colon and cause an intestinal amebiasis. The trophozoites multiply by binary fission and produce cysts. Both trophozoites and cysts are passed in the feces. The cysts can survive days to weeks in the external environment and are responsible for transmission because of the protection conferred by their walls. However, unlike cysts, trophozoites passed in the stool are rapidly destroyed once outside the body, and if ingested would not survive

exposure to the gastric environment. The trophozite can also invade the large intestinal wall and cause extraintestinal amebiasis when the trophozites invade blood vessels and hematogenously spread to other organ such as brain, lung and liver. In these organs, the trophozoites will feed on the cells and subsequently lead to the formation of abscess in the invaded organ.

In many cases, the trophozoites remain confined to the intestinal lumen. Some infected individuals may not show any symptom but become an asymptomatic carrier and passing cysts in their stool. The 'carrier' or asymptomatic human host is responsible for continuously passing out the infective stage cysts to the environment (Figure 1.1).

1.2 Epidemiology of amoebiasis

Prior to the differentiation of *E. histolytica* from its non-pathogenic sibling *Entamoeba dispar* in 1993, amoebiasis was the second major parasitic diseases after malaria and it was responsible for approximately 100,000 human deaths per year (Das & Ganguly, 2014). According to WHO (1997), amoebiasisis prevails in all developing countries and at times its prevalence reaches up to 50% of general population.



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

Figure 1:1 Life cycle of *E. histolytica.*

Asymptomatic infections with *E. histolytica* are common, whereas the symptoms of invasive amoebiasis develop in approximately 10% of the infected individuals, resulting in 50 million cases and 100,000 deaths annually. Recent data on prevalence and morbidity suggested that amoebiasis is probably endemic in many less-developed countries which areas are poor of sanitation and hygiene (Haque *et al.*, 2003; Pritt *et al.*, 2008; Farhana *et al.*, 2009; Kuhswaha *et al.*, 2012; Tan *et al*, 2012) making this disease a permanent public health problem that needs attention from health authorities.

Currently, species-specific information on *Entamoeba* infections is not available in Malaysia and is restricted worldwide due to the re-description of pathogenic *E. histolytic*a and non-pathogenic *E. dispar* and *Entamoeba moshkovskii*. According to a molecular epidemiology study of amoebiasis among Orang Asli communities, molecular analysis revealed that most *Entamoeba*-positive individuals were actually infected with *E. dispar* (13.4%), followed by *E. histolytica* (3.2%) and *E. moshkovskii* (1.0%) (Anuar *et al.*, 2012).

1.3 Pathogenesis of *E. histolytica*

The name *E. histolytica* itself suggests the destructive nature of the protozoon. The term 'histolytic' means a tissue destroyer. *E. histolytica* can lyse and destroy human tissue. ALA is defined as a circumscribed region of dead hepatocytes, liquefied cells and cellular debris surrounded by a rim of connective tissue, some inflammatory cells and few amoebic trophozoites (Sharma, 2012)

E. histolytica binds host cells via galactose-bining lectin on the surface of the amoeba. Once contact is made, amoeba releases pore-forming molecules call amoebapores which can cause lysis of the cells (Stanley, 2001). Cell lysis might then be followed by amoebic phagocytosis of the dead cell.

According to the *in-vitro* study by Stanley (2001) it was found that neutrophils, which are the most prominent inflammatory cell type seen early in amoebic infection can be lysed by *E. histolytica* trophozites *in vitro*, causing them to release toxic substances such as superoxides, collagenases, elastases and cathepsins. In a study of the interaction between amoebic trophozoites and an epithelial cell monolayer, the addition of neutrophils to ameobic trophozoites significantly increased damage to the monolayer. This eventually leads to the invasion of trophozoites through the mucosa, and blood vessels, particularly the hepatic portal vein where it can travel by blood circulation to reach the liver and causes lesion.

1.4 Diagnosis of *E. histolytica* infection

In diagnosis of *E. histolytica* infection, the pathogen must be differentiated from other intestinal protozoa which mostly are the non-pathogenic amoebas such as *E. coli*, *E. hartmanni*, *E. gingivalis*, *Endolimax nana*, and *Iodamoeba buetschlii* and the possibly pathogenic *Entamoeba polecki* (Parija, 2003). Microscopic stool examination for trophozoites is only 33-50% sensitive from a stool sample in amebic colitis and the results for stool examination findings in patients with amebic liver abscess are usually negative (Lim *et al.* 2014). Differentiating *E. histolytica* from other intestinal protozoa based on morphologic characteristics of the cysts and trophozoites can sometimes be difficult. Moreover, the nonpathogenic *E. dispar* is morphologically identical to *E.*

histolytica, and differentiation must be based on isoenzymatic, immunologic or molecular analysis.

1.4.1 Problems in current diagnosis of *E. histolytica* infection

Current diagnosis of ALA is typically based on the clinical symptoms which are often described as a constant dull, aching abdominal pain in the right upper quadrant (Haque *et al.*, 2003), abscess detected by radiology imaging, and serology. However, radiology imaging cannot differentiate between amoebic and pyogenic abscesses. On the other hand, current antibody serology that use the commercial IHA kit cannot differentiate between present and past infections and may also reveal false positivity and thus not specific in diagnosis due to the use of crude soluble antigen (CSA) preparation consisting cocktail antigen of *E. histolytica* (Tan *et al.*, 2013).

The current means for diagnosis of ALA is the detection of anti-amoebic antibody by serological tests combined with aspiration of the abscess. The presence of serum antibodies against *E. histolytica* and the absence of bacteria in the abscess fluid are consistent with an ALA. A further limitation to the current approach to ALA diagnosis is that collection of liver abscess pus is an invasive procedure that requires technical expertise and can be done only in specialized hospitals.

1.5 Rationale of the study

1.5.1 Previous work of this project

Previously, in a study by Lim *et al.* (2014), *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. Protease inhibitor was added to inhibit proteases activities. In a 2-D Western blot analysis against the ALA serum samples, the antigenic protein band obtained was sent for sequencing. Based on the sequencing result, acetyl-CoA synthetase was confirmed to be the antigenic protein associated with the acute infection.

Molecular cloning was then performed by extracting the genomic DNA of the trophozoites 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 specificity and sensitivity of r*Eh*ACS against ALA hamster antibodies was evaluated and Western blot analysis showed that the sensitivity and specificity of r*Eh*ACS were 58.07% and 100% respectively; while the sensitivity and specificity of the recombinant *Eh*ACS used in an ELISA format were both 100% (Lim *et al.*, 2014).

This study was done to evaluate the specificity and sensitivity of this recombinant protein (r*Eh*ACS) against ALA human antibody in r*Eh*ACS/total anti-human IgG and r*Eh*ACS/anti-human IgG_4 ELISA format.

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

Recombinant *Eh*ACS was identified as a potential biomarker in hamster experimentation by Lim *et al.* (2014). The sensitivity and specificity of r*Eh*ACS was evaluated using hamster ALA serum sample in an indirect ELISA format. Approximately ~77kD protein band was detected by all hamster ALA serum sample in Western Blot analysis. A customized ELISA result revealed 100% sensitivity and 100% specificity when tested against infected (n=31) and control group (n=5) hamster serum sample. In experimentally induced hamster ALA, there was massive death of trophozoites during the first few hours post-infection (Faust and Guillen, 2012). It is suggested that *E. histolytica* antigens, including *Eh*ACS, released from these dead amoebas triggered the host humoral responses during this early stage of infection. The 12 h post-infection period was appointed to be the critical stage for successful invasion by the amoebas into the hamster liver, which the lowest number of trophozoites was observed in the infected liver. The live amoebas probably continued to secrete or excrete *Eh*ACS that boosted the production of antibody, or antibody production was due to release of the highly immunogenic antigen during subsequent death of trophozoites (Lim *et al.*, 2014).

1.6 Objectives of the study

1) To evaluate the potential of r*Eh*ACS for detection of ALA in human serum samples
2) To develop and compare the efficiency r*Eh*ACS/IgG-ELISA and r*Eh*ACS/IgG$_4$-ELISA for detection of human ALA

CHAPTER 2

MATERIALS AND METHODS

2.1 Culture test on *E. coli* BL21 A1 and BL21 A1 with pET-14b/ACS

In this study, recombinant *Eh*ACS was over-expressed in *E. coli* BL21 AI containing pET-14b/ACS plasmid. Since the bacterial culture was obtained from a previous work, a culture test was performed to determine whether the recombinant bacteria can work ideally. Over-expression on small amount of culture was performed, followed by affinity purification and analysis by SDS-PAGE.

2.1.1 Preparing overnight culture

The culture plates containing the bacterial culture were taken out from the 4°C refrigerator and incubated at 36°C for 10 minutes. In a sterilized working area, the *E.coli* BL21 A1 with pET-14b/ACS was sub cultured onto a new Luria-Bertani (LB) agar supplemented with ampicillin (100μg/ml) by streaking method. *E. coli* BL21 A1 was also subcultured on LB agar supplemented with ampicillin and another LB agar without ampicillin for negative and positive control respectively. All the culture plates were sealed with parafilm and incubated in the incubator at 32°C overnight. The next day, the overnight culture was observed. All the overnight culture was sub-cultured again if colonies of *E.coli* BL21 A1 grow on the LB agar supplemented with ampicillin.

2.1.2 Protein over-expression

Overnight culture was prepared by transferring one bacterial colony from the culture plate into 5ml LB culture medium (pH 7.0) supplemented with ampicillin

(100µg/ml). The tube was shaken at 200 rpm overnight at 37°C. On the next day, 1 mL of the overnight culture was transferred into a conical flask containing 50 ml of LB medium supplemented with ampicillin (100 µg/mL) and incubated for 2 hours at 180 rpm in a shaker incubator, at 36°C, until the optical density (OD_{600}) reached 0.6 – 0.8 which takes about 2 hours. After 2 hours, 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. At OD 0.65, over expression was induced by adding 500 µL of 20% L-arabinose was added into the cell suspension. The cell suspension was incubated in a shaker incubator at 180 rpm overnight or 16 hours at 36°C.

On the following day, the OD for post-induced over expression was checked using Bradford method. One mL of the post-induced culture was transferred into 1.5mL microcentrifuge tube; centrifuged at 10, 000 × *g* for 5 min, 4 °C; and the cell pellet was kept at -80 °C for later analysis. The cell suspension was poured into 50mL Falcon tube and centrifuged at ~2000 rpm for 10 minutes at 4°C. The supernatant was discarded and the cell pellet was resuspended in 5mL 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 2 min cycles at 20 % 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 centrifuge at ~2000rpm and 4°C for 10 minutes. Hundred µL of the supernatants and the pellets were transferred into 1.5mL microcentrifuge tube and kept separately at -80 °C for later analysis.

2.1.3 Protein purification

His-Tag recombinant *Eh*ACS 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 1 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 r*Eh*ACS protein. Four hundred µL of the supernatant was mixed with the washed resin and incubated 1 hour in cold room (4 °C) with continuous rotation.

The resin with captured r*Eh*ACS protein was washed with washing buffer containing 80 mM imidazole. 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. 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.

2.1.4 Analysis of protein purify via SDS-PAGE

Expressed protein was analysed via 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). 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.

Small gel [8 cm (W) X 7.3 cm (H) X 0.75 mm (T) made of 10% SDS-PAGE resolving gel is prepared for analysis of protein purify using 2.5 mL 4× resolving buffer, 4.79 mL distilled water, 2.5 mL acrylamide (40%), 0.1 mL SPS (10%), 0.1 mL APS (10%) and 0.01 mL TEMED. 3% stacking gel was prepared using 1.25 mL 4× stacking buffer 3.27ml distilled water, 0.375 mL acrylamide (40%), 0.05 mL SPS (10%), 0.05 mL APS (10%) and 0.005 mL TEMED. A thin layer of isopropanol was added to the gel.

The protein samples were mixed with 2X sample buffer in the ratio of 1:1 and boiled at 95°C for 5 min. The boiled samples were then centrifuged at 10, 000 rpm for 10 minutes and the supernatant from each sample was loaded into the wells of stacking gel. The electrophoresis was run at a constant current with 0.02 A and 120 V for 40 minutes or until the Bromophenol blue dye was ~0.5 cm from the bottom of the glass plate. Upon completion of the electrophoresis, the stacking gel was detached and removed from the resolving gel. The resolving gel was carefully removed from the glass plate and was stained in CBB (Bio Basic, Canada).

Table 2.1 Gel casting ingredient for SDS-PAGE

Ingredient	**Resolving Gel (10 %)**	**Stacking Gel (3 %)**
Resolving Buffer, pH 8.8	2.5 mL	-
Stacking Buffer, pH 6.8	-	1.25 mL
Acrylamide, 30 %	2.5 mL	0.375 mL
SDS, 10 %	100 µL	50 µL
APS, 10 %	100 µL	50 µL
TEMED	10 µL	5 µL
dH2O	4.79 mL	3.27 mL
Total	**10 mL**	**5 mL**

2.2 Optimization of r*Eh*ACS-ELISA

2.2.1 Optimization for ELISA analysis

The ELISA plate was first labeled with PBS, positive control and negative control for two sets of r*Eh*ACS-ELISA for total anti-human IgG and anti-human IgG_4. The r*Eh*ACS antigen was prepared by adding 4ml coating buffer into 25µL antigen. All the wells are added with 100µL coating buffer except for the first row. The wells in the first and second row were each added with 100 µL of the prepared antigen. Serial dilution was performed from the second well to the eighth well and the last 100 µl dilution was discarded. Antigen will be diluted by half on subsequent row (20, 10, 5, 2.5, 0.625,..., 0.15625µg/mL). The coated ELISA plate was incubated in a moist chamber at -4°C overnight.

On the following day, the plate was incubated at 100 rpm on a plate shaker at room temperature for 10 minutes. Blocking reagent was prepared by diluting 2 mL of 10X blocking reagent in 20 mL 1X phosphate buffer solution (PBS). Next 0.005% PBS-T solution was prepared by adding 500 µL Tween 20 on 1000 mL PBS. The antigen was discarded and washed with 200 µL Phosphate-buffered saline with 0.05 % Tween 20 (PBST) (3 X 5 min) on the shaker (200 rpm) at room temperature. Next, the wells were incubated with 200 µL blocking buffer for one hour at room temperature and washed three times with PBST.

The wells were incubated with 100 µL of diluted patient's pooled antibody (primary antibody) (1:100) for one hour on the shaker (200 rpm) at room temperature. The wells were next washed with 200 µL of PBST (3 X 5 min) on shaker (200 rpm).

After primary antibody, the wells were incubated with diluted monoclonal antibody (secondary antibody) (Sigma, USA) (1:1000) for one hour on a shaker (200 rpm) at room temperature and then washed three times with PBST for 5 min on a shaker (200 rpm) at room temperature. Blue colour was developed after 100 µL of TMB substrate (Sigma, USA) added in. Then, the plate was put in a dark room for 15 min. Colour was developed from blue to yellow after 100 µL of stop solution added in. The result was read by the ELISA Reader and a graph was developed to determine the optimized antigen concentration.

The optimization step was repeated for determining the optimum primary antibody concentration and secondary antibody concentration. For the next optimization, the result from the antigen concentration optimization was used to determine the concentration of r*Eh*ACS antigen to be used to coat the ELISA well in later analysis and optimization. The primary antibody concentration optimization was performed with serial dilution by half on subsequent row (1:25, 1:50, 1:100,...., 1:3200)

2.3 ELISA analysis

2.3.1 ELISA assay

A 96 wells microtiter plate was used to run the ELISA assay. One hundred µL of 20 µg/mL (determined from Section 2.2) purified protein was added into each well and incubated overnight at 4 °C. On the next day, incubation was carried out again at room temperature with shaking (200 rpm) for one hour. Then the plate was washed 3 times with 200 µL of PBST for 5 min on a shaker (200 rpm). Next, the wells were incubated with 200 µL blocking buffer for one hour at room temperature and washed three times with PBST.

The wells were incubated with 100 µL of the diluted primary antibody (1:100) for one hour on the shaker (200 rpm) at room temperature. These primary antibodies include pooled ALA positive serum as positive control, pooled ALA negative serum as negative control, ALA positive patient serum and ALA negative serum. The wells were next washed three times with 200 µL of PBST for 5 min on the shaker (200 rpm). After the primary antibody, the wells were incubated with the diluted secondary antibody (1:500) for one hour on the shaker (200 rpm) at room temperature and then washed three times with PBST for 5 min on the shaker (200 rpm) at room temperature. Blue colour was developed after 100 µL of TMB substrate added in. Then, the plate was put in a dark room for 15 min. Colour developed from blue to yellow after 100 µL of stop solution added in. The result was read with the ELISA Reader and the specificity and sensitivity of r*Eh*ACS against the human serum samples were determined based on the cut-off value (COV) displayed on a graph.

2.3.2 Determination of COV

COV was calculated by using the formula below:

$$\text{COV} = \text{Mean of negative control} + 2\text{SD}$$

2.3.3 Determination of sensitivity

In this study, the sensitivity was defined as the ability of the antibody to detect the presence of recombinant ACS protein in the wells. This is determined by using the formula below:

$$\text{Sensitivity} = \frac{\text{N(true positive)}}{\text{N(ALA positive serum)}} \times 100\ \%$$

2.3.4 Determination of specificity

Specificity is defined as the ability of the recombinant ACS to identify those free of disease in the ALA negative serum and was calculated as below:

$$\text{Specificity} = \frac{\text{N (true negative)}}{\text{N (ALA negative serum)}} \times 100\ \%$$

CHAPTER 3

RESULTS

3.1 Overnight culture on LB agar plate

BL21A1 containing recombinant pET-14b/r*Eh*ACS was successfully cultured on LB agar plate supplemented with 100μg/mL ampicilin (Figure 3.1A). The positive and negative controls also produced expected results (Figure 3.1B and 3.1C).

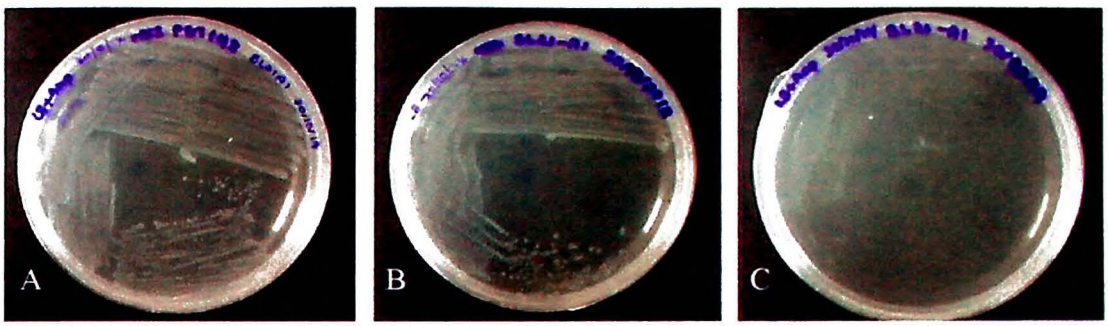


Figure 3.1(A) Expression bacteria BL21A1 containing recombinant pET-14b/r*Eh*ACS colonies grow on LB agar plate supplemented with 100μg/mL ampicillin.

(B) BL21A1 colonies grow on LB agar plate without ampicillin. This is the positive control.

(C) BL21A1 colonies did not grow on LB agar plate supplemented with 100mg/μL ampicillin. This is the negative control.

3.2 Analysis of protein purity via SDS-PAGE

The recombinant *Eh*ACS with molecular weight of ~77 kDa was successfully produced (Figure 3.๑). Optimized temperature and incubation duration that was used to produce the r*Eh*ACS has been described by Lim *et al.* (2014).

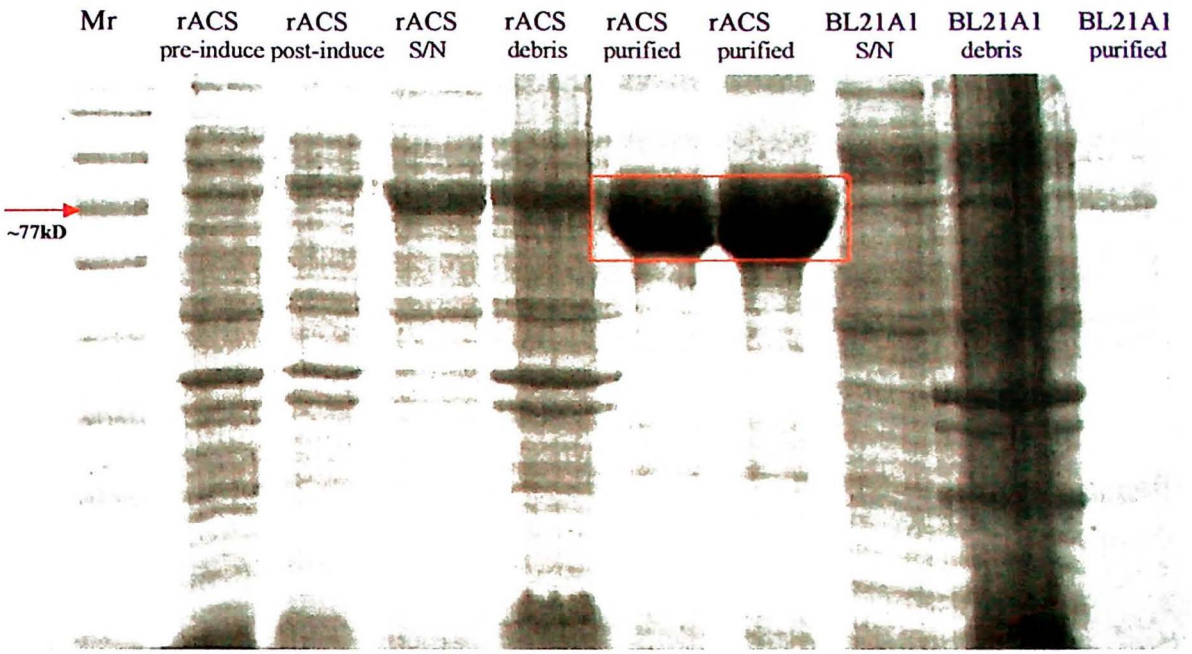


Figure 3.2 The recombinant *Eh*ACS with molecular weight of ~77kD was successfully produced based on band shown at ~77kD when compared to the marker.

Note: The box show the band of ~77kD of purified recombinant *Eh*ACS protein.

3.3 ELISA analysis

3.3.1 Optimization of r*Eh*ACS antigen concentration

Based on Figure 3.3, the red and pink lines which represented the positive serum sample showed fluctuation in OD_{450} before 20 µg/mL. At 20 µg/mL, the line showed almost a straight line. The antigen concentration of 20 µg/mL was determined to be used later. The green and blue lines represented the negative serum sample and PBS respectively. These lines are almost straight line except for ALA negative serum tested against IgG_4 which means that no reaction occurred in these wells.

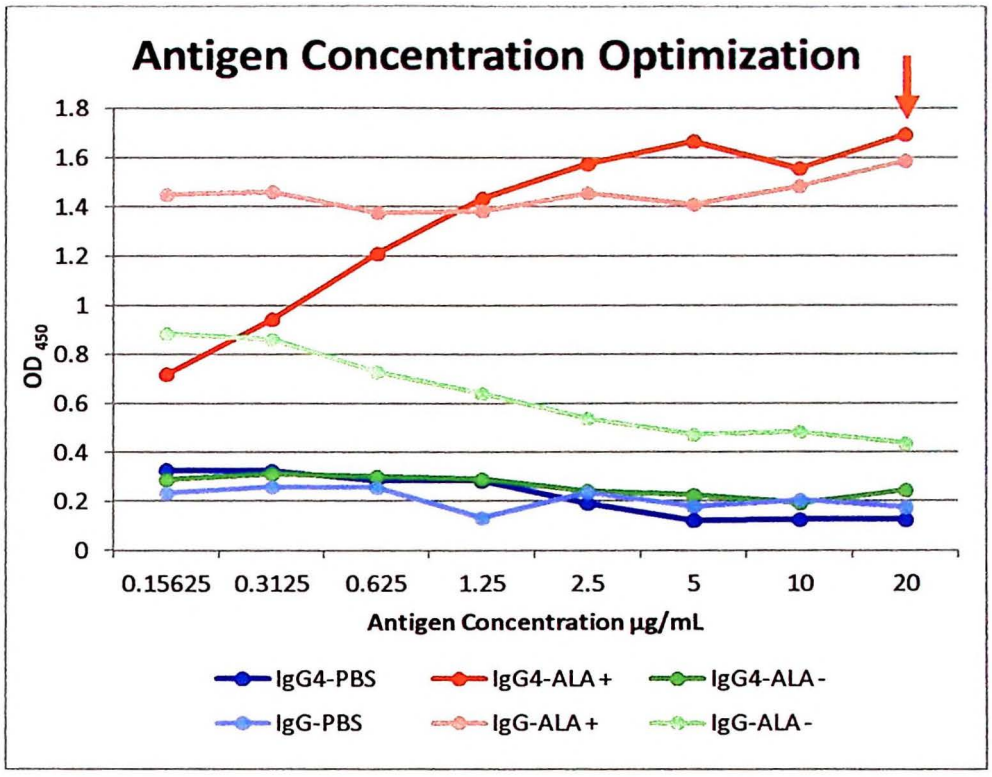


Figure 3.3 Optimization of antigen concentration by using ELISA Assay. PBS: Phosphate-buffered saline; ALA+: ALA positive patient serum samples; and ALA-: IHA seronegative serum.