COMPARISON OF THE EFFICIENCIES OF CUSTOMIZED CSA- AND rLECTIN-ELISAS FOR DETECTION OF ANTI-AMOEBIC ANTIBODY BASED ON SELECTED ABORIGINES SERUM SAMPLES

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By

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CERTIFICATE

This is to certify that the dissertation entitled **COMPARISON OF THE EFFICIENCIES OF CUSTOMIZED CSA- AND rLECTIN-ELISAS FOR DETECTION OF ANTI-AMOEBIC ANTIBODY BASED ON SELECTED ABORIGINES SERUM SAMPLES** is the bona fide record of research work done by **ABEER MUATAZ ABED ALTAMEEMI** during the period from February 2017 to December 2017 under my supervision.

Supervisor,

_____

ASSOCIATE PROFESSOR DR. FEW LING LING

Date: _____

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMNS

~	About
%	Percentage
>	More than
^{0}C	Degree Celsius
µg	Microgram
µL	Microliter
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
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
r*Eh*ACS	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

PERBANDINGAN ANTARA KEBERKESANAN ELISA-CSA DAN ELISA-rLECTIN YANG DISESUAIKAN DALAM PENGESANAN ANTIBODI ANTI-AMEBA DALAM SAMPEL SERUM ORANG ASLI YANG TERPILIH

ABSTRAK

Orang Asli Malaysia tinggal di hutan pedalaman yang endemik terhadap amebiasis. Pemahaman terhadap prevalens dan taburan penyakit ini adalah penting untuk rancangan kawalan dan pencegahan. Salah satu penunjuk utama untuk penyakit ini adalah kehadiran antibodi anti-ameba yang sangat tinggi. Walau bagaimanapun, penyaringan berterusan antibodi menggunakan kit komersil adalah tidak praktikal kerana penglibatan kos yang tinggi. Kit buatan sendiri yang lebih murah dan memberi keputusan yang sahih harus diguna sebagai pengganti untuk menjimat kos dan kelestarian. Oleh yang demikian, kajian ini bertujuan untuk membandingkan keberkesanan ELISA-CSA (antigen larut kasar) dengan ELISA-rLectin (lektin rekombinan) dalam pengesanan antibodi anti-ameba yang terkandung dalam sampel serum Orang Asli yang telah terlebih dahulu diuji dengan kit hemaglutinasi secara tidak langsung (IHA) komersial. CSA adalah dihasilkan daripada trofozoit *Entamoeba histolytica* yang dikultur secara axenik, sementara rLectin diekspress dan ditulenkan daripada *Escherichia coli* yang mengandung gen rekombinan itu. Pembangunan kedua-dua ELISA itu melibatkan pengoptimuman kepekatan antigen, kadar pencairan serum dan antibodi yang dikonjugasi. Keberkesanan diagnostik kedua-dua ELISA itu dibanding dengan menggunakan 30 sampel serum positif dan negatif yang telah ditentukan oleh asai IHA komersial. Berdasarkan analisis lengkung ciri operasi penerima (ROC curve), kedua-dua ELISA itu menunjukkan prestasi diagnostik yang

sangat baik. Apabila dibanding dengan ujian rujukan, persetujuan peratusan positif ELISA-CSA (91%) didapati lebih tinggi daripada ELISA- rLectin (61%), manakala kedua-dua ELISA itu menunjukkan persetujuan peratusan negatif yang sama iaitu 97%. Menurut analisis densiti optik ELISA, nilai delta positif ELISA- CSA (0.4472) adalah lebih tinggi daripada nilai delta positif –ELISA-rLektin (0.2673), manakala nilai delta negatif ELISA-rLektin (-1.209) lebih rendah daripada nilai delta negatif ELISA-CSA (-1.175). Kesimpulannya kajian ini menunjukka bahawa ELISA-CSA adalah lebih sensitif dalam mengesan kehadiran antibodi anti-ameba, sementara ELISA- rLectin lebih spesifik dalam menentukan kes-kes negatif.

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ABSTRACT

Orang Asli, the Malaysian aborigines live in remote jungles, are endemic for amoebiasis. Understanding the disease prevalence and distribution is important for the control and preventive measures. A major indicator that reflects on the disease incidence is the presence of highly elevated anti-amoebic antibody. However, continuous screening of the antibody using commercial kits is impractical due to the high cost. Cheaper but valid in-house customized assay should be employed instead to attain cost-effective and sustainable outcome. Hence, the presence study aimed to compare the efficiencies of customized crude soluble antigen (CSA)-ELISA and recombinant lectin (rLectin)-ELISA for detection of anti-amoebic antibody in selected Orang Asli serum samples, which were prior defined by commercial indirect haemagglutination assay (IHA). CSA was produced from axenically grown *Entamoeba histolytica* trophozoites, while rLectin was overexpressed and purified from *Escherichia coli* harbouring the corresponding recombinant gene. For the development of the two customized ELISAs, antigen concentration, serum and conjugated antibody dilutions were optimized. The efficiencies of both customised ELISAs were determined by 30 positive and negative serum samples pre-determined by commercial IHA. According to receiver operating characteristics (ROC) curve analysis, both customized ELISAs showed excellent diagnostic performances. As compared to the reference assay, the positive percentage agreement of CSA-ELISA (91%) was higher than that of rLectin-ELISA (61%), while

both ELISAs showed the same negative percentage agreement, which was 97%. According to analysis of ELISA ODs, the positive delta value for CSA-ELISA (0.4472) was higher than that of rLectin-ELISA (0.2673), while the negative delta value of rLectin-ELISA (-1.209) was lower than that of CSA-ELISA (-1.175). In conclusion, this study demonstrated that CSA-ELISA was more sensitive in detecting the presence of anti-amoebic antibody, while rLectin-ELISA was more specific in ruling out negative cases.

CHAPTER ONE

INTRODUCTION

1.1 *Entamoeba histolytica*

Amebiasis is an infection caused by *E. histolytica*, the protozoan parasite, which is the third leading cause of death worldwide after malaria and schistosomiasis (Huston & Sateriale 2011; Shahrul Anuar et al., 2012). There are six species in the genus of *Entamoeba* which are found in the intestinal lumen of humans, namely *E. histolytica*, *Entamoeba dispar*, *Entamoeba moshkovskii*, *Entamoeba coli*, *Entamoeba polecki* and *Entamoeba hartmanni*. Among them, *E. histolytica* is the only species that causes invasive disease resulting in diarrhoea, dysentery and liver abscess in human. Based on microscopic observations, all nuclei of *E. histolytica*, *E. dispar* and *E. moshkovskii* have a central karyosome, which differentiated them from the other non-pathogenic *Entamoeba* species. Differentiation between the pathogenic *E. histolytica* from the two other non-pathogenic but morphologically similar *Entamoeba* is important for treatment purposes. The recent development of molecular diagnostics enables the differentiation among the three *Entamoebas*. *E. moshkovskii* is primarily considered to be a free-living ubiquitous amoeba found in anoxic sediments. Recently, a new species of *E. bangladeshi* was discovered but its importance to human health is basically unknown. It was found to be genetically more closely related to *E. moshkovskii* than *E. histolytica* (Lau *et al.*, 2013).

1.1.1 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. The other amoeba species are important because they may be confused with *E. histolytica* in diagnostic investigations. The infection occurs usually in the large intestine and causes inflammation or abscess in other organs such as liver. The most common form of organ abscess caused by *E. histolytica* is amoebic liver abscess (ALA). The protozoan parasite life cycle consists of two forms, namely an infective cyst form and a motile pathogenic trophozoite form.

The cycle begins when human accidentally ingests food or drink contaminated with *E. histolytica* cyst (Figure 1.1). Each cyst transforms into four trophozoites through a process called excystation in the small intestine. The trophozoites multiply by binary fission and then transform into cysts. Both trophozoites and cysts are passed in the faeces. Cysts are typically found in formed stool, whereas trophozoites are typically found in diarrheal stool. The cysts can survive for days to weeks in the external environment and are responsible for transmission. The cysts are conferred protection by the presence of cyst walls. However, trophozoites that are passed in the stool are rapidly destroyed once outside the body as they do not have cyst walls. If the trophozoites were ingested, they would not survive in the human gastric environment. In many cases, the trophozoites remain confined to the intestinal lumen. Some patients may not show any symptom and they became asymptomatic carriers who pass cysts in their stool. In some patients the trophozoites invade the intestinal mucosa (intestinal disease), enter the bloodstream and spread to other organs such as the liver, brain, and lungs which result in extraintestinal diseases such as ALA.

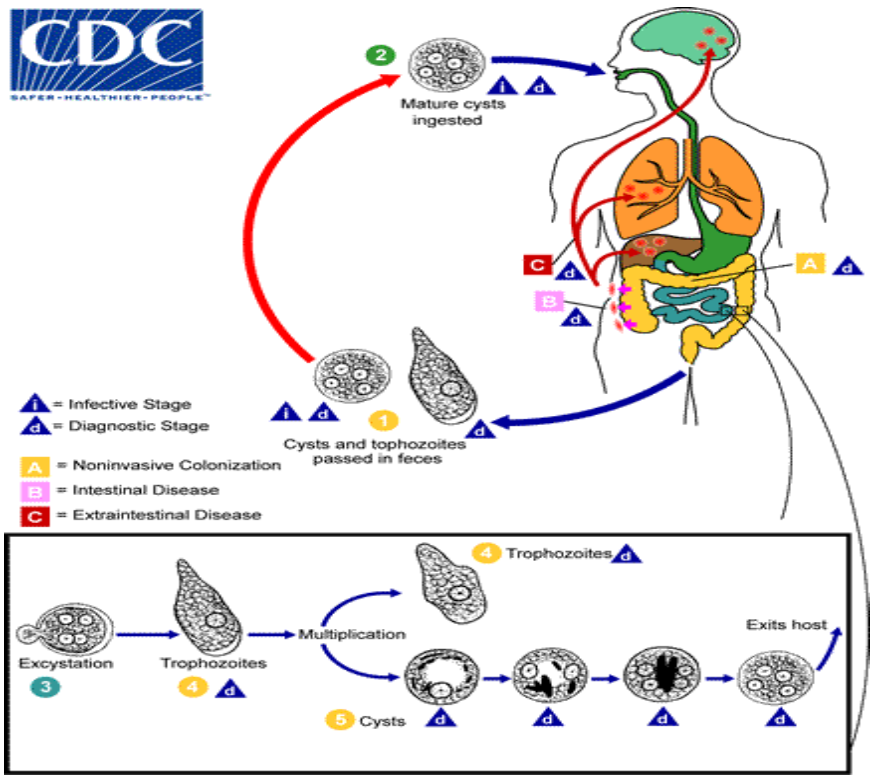


Figure 1.1: Life cycle of *E. histolytica* (CDC 2015)

1.1.2 Morphology

E. histolytica trophozoite has a diameter of 12-60 µm and is surrounded by a three-tiered, lipid-protein cell membrane and creates characteristic ameboid pseudopodia that allow it to move and participate in phagocytosis, a process of absorption of food particles. The cytoplasm is differentiated into ectoplasm and fine-grained endoplasm, which consists of cytosol and numerous cell organelles such as endosomes, lysosomes, Golgi apparatus, vacuoles with red blood cells and glycogen mass. *E. histolytica* does not contain mitochondria, which is why it obtains its source of energy via anaerobic process by releasing glucose from the stored glycogen mass. Trophozoite of *E. histolytica* contains one round nucleus, in which the genetic material (DNA) is concentrated in the form of a small, dense, centrally located karyosome and peripherally, evenly deployed chromatin. The shape and position of the karyosome and the placement of chromatin in the cell nucleus is the characteristic of *E. histolytica*, which is used in their differential diagnosis. *E. histolytica* trophozoite secretes specific proteolytic enzymes such as hyaluronidase, cysteine proteinase that can degrade and lyse human cells and tissues. The image of *E. histolytica* trophozoite is shown in Figure 1.2.

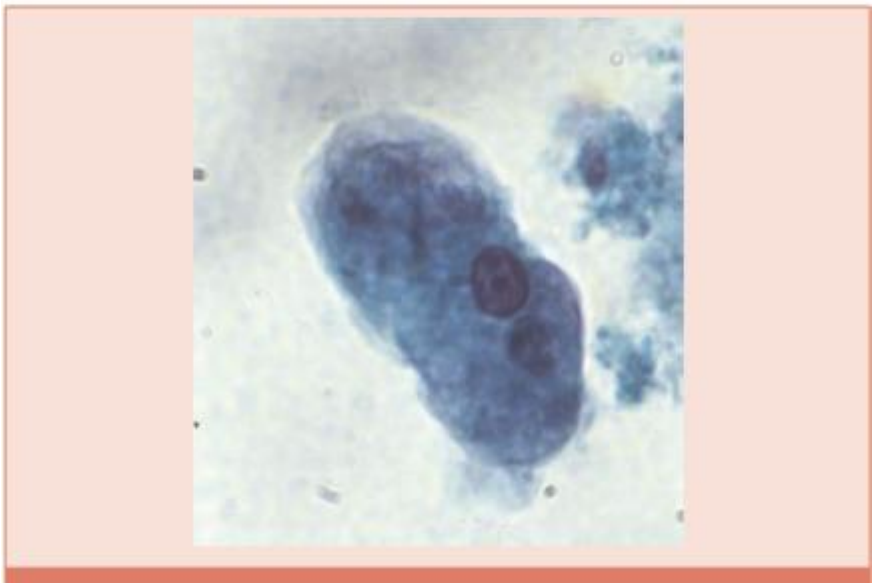


Figure 1.2: Trophozoite stage of *E. histolytica* (Technology, 2015)

The cyst of *E. histolytica* is the infective stage. It can survive in adverse conditions in the external environment for many days. The cyst has a diameter of 10-20 μm. It is usually round and contains 1, 2, 3 or 4 nuclei with karyosome peripherally placed chromatins. A mature cyst of *E. histolytica* usually contains irregular shaped glycogen in its cytoplasm which is stained dark or orange-brown with Lugol's iodine. In addition, the cyst also contains blunt finished chromatoidal bars. These structures are clearly visible in preparations stained with iodine or Trichrome (Gömöri-Wheatley technique). Figure 1.3 shows the cyst *E. histolytica* (Technology, 2015).

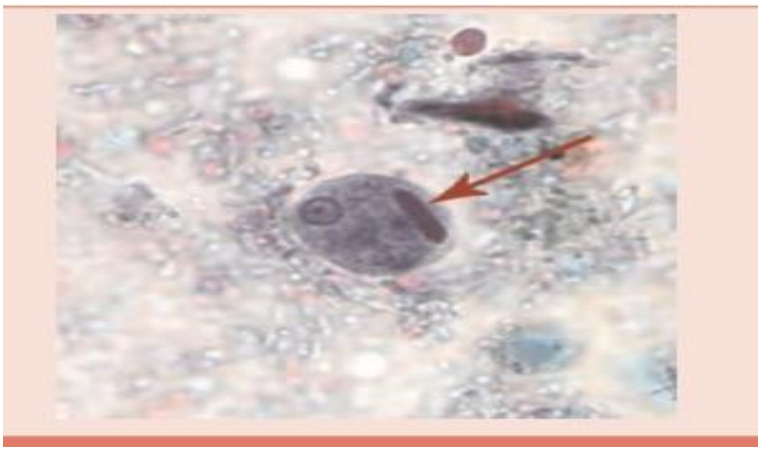


Figure 1.3: Cyst stage of *E. histolytica* (Technology, 2015)

1.2 Epidemiology of Amoebiasis

1.2.1 Geographical Distribution of Amoebiasis

Amoebiasis, an infection by protozoa *E. histolytica* is appraised as the third leading parasitic cause of human mortality after malaria and schistosomiasis (Nath *et al.*, 2015). Amoebiasis accounted for 40,000 to 100,000 cases of death each year. Only 10 to 20% of the persons infected by *E. histolytica* developed the symptoms and manifested as amoebiasis. Significant progress has been made on the classification of *Entamoeba* in which the existent of two identical species were confirmed, namely the pathogenic *E. histolytica* and non-pathogenic *E. dispar*. According to the report released by The Pasteur Institute in 2012, in some tropical regions, the prevalence in *E. histolytica* may even reach 20% of the population. One percent of world

population is infected by *E. histolytica*; this is why *E. histolytica* is the second leading cause of mortality among the human parasites. Amoebiasis is genuinely a major handicap to the health of the people under fairly poor hygienic conditions and primarily living under the poverty line, mostly in the developing countries like India, Bangladesh and Mexico. In Africa surveys and researches conducted in Sudan, Ivory Coast, Ethiopia, Nigeria, Egypt and South Africa revealed the fact that in some local regions the prevalences of amoebiasis were high. On the contrary, rare cases were reported in developed countries like United States of America (USA) and Western European countries (Hategekimana, Saha & Chaturvedi, 2016).

1.2.2 Distribution of Amoebiasis in Malaysia

The prevalences of *E. histolytica, E. histolytica/E. dispar/E. moshkovskii* among the Orang Asli communities in Malaysia have been reported to range from 1% to 83% by many researchers since the 1960s. Most of the reports did not take into considerations that the three amoeba species were morphologically identical and cannot be distinguished by microscopy. Recently the separate prevalences of *E. histolytica* and *E. dispar* among the Orang Asli communities were estimated at 13.2% and 5.6%, respectively. These were determined based on the re-description of pathogenic *E. histolytic*a and non-pathogenic *E. dispar* and *E. moshkovskii* (Anuar *et al.*, 2012).

1.2.3 Reservoir, Source and Transmission of Amoebiasis

Amoebiasis caused by the protozoan parasite *E. histolytica*. According to WHO, transmission of amoebiasis occurs via the faecal–oral route, either directly by person-to-person contact or indirectly by eating or drinking faecally contaminated food or water with cyst of *E. histolytica* (World Health Organization, 2016).

1.3 Pathogenesis of Amoebiasis

E. histolytica can be found mainly in countries, areas, and institutions with poor sanitation and water systems contaminated with cysts of *E. histolytica* (World Health Organization 2016). Acid in human stomach kills *E. histolytica* trophozoites but not the cysts. In fact the cysts can resist the strong stomach acid and excyst into trophozoites in the lumen of the small intestine.

Successful invasion of *E. histolytica* on host depends on the parasite abilities to cross the intestinal or hepatic barrier, resist and escape the host responses, destroy the tissue and migrate to the host microenvironment. Hence various diverse factors have to be coordinated for a successful invasion of its host. Some important molecules have been studied for their roles in tissue invasion: adherence, cytotoxicity, cell killing and phagocytosis as well as the onset of host immune responses (Faust &Guillen, 2012). The invading trophozoites may cause asymptomatic luminal colonization, amebic colitis and amebic liver abscess is *E. histolytica* (Petri Jr & Haque 2015).

The initial contact of the trophozoite to host luminal wall is mediated by *E. histolytica* Gal/GalNAc lectin, which binds to carbohydrate determinants on the wall.

With the aid of amebic lectin, cytolysis occurs after adherence in the process that involves phagocytic ingestion of the host cells in bites, called “trogocytosis-like”. Figure 1.4 shows the process of cytolysis. However, killing of host cells is not caused by an isolated toxin, inasmuch as parasite extracts have no cytotoxic activity. Lastly, in multicellular organisms, phagocytosis is the final step in the apoptotic pathway and serves to limit inflammation by preventing spillage of toxic intracellular contents of dead cells. Although amoebic killing of cells by contrast involves phagocytosis followed by death, phagocytosis could similarly limit the host inflammatory response and enable *E. histolytica* to establish a persistent infection.

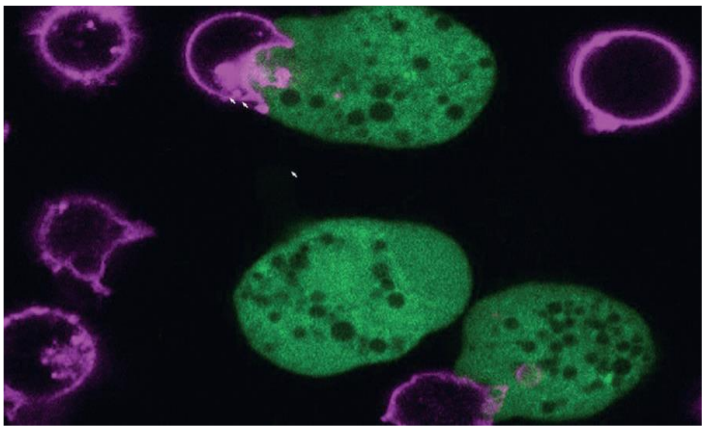


Figure 1.4: Trogocytosis-like killing of host cells by *E. histolytica* [Scientific American (2014). Obtained on 18 August 2017 at https://www.scientificamerican.com/article/amoeba-takes-bites-of-human-cells-to-kill-them/].

Human colon produces mucin which binds to and inhibits *E. histolytica* Gal/GAlNAc adherence lectin. However, *E. histolytica* encodes for at least 44 cysteine proteinases genes, in which some of the cysteine proteases are reported to be potentially important for the degradation of colonic mucin glycoproteins and digestion of hemoglobin and villin of intestinal epithelial cells (Mandell, Douglas & Bennett 2015).

1.4 Host Immunity

1.4.1 Innate immunity:

E. histoytica trophozoite is a microaerophilic protozoan which is killed in host environment with high oxygen content such as in the presence of reactive oxygen species (ROS) and nitirc oxide (NO).

Neutrophils are the earliest innate cellular immune response for both intestinal and hepatic amebiasis. It is one of the first immune cells to respond to amoebic invasion. As a consequence of interaction with *E. histoytica* trophozoites, neutrophils become activated and release reactive oxygen species (ROS) which are toxic to the trophozoites. Besides neutrophils, macrophages produce NO to kill the trophozoites when they are stimulated with INF-gamma or TNF-alpha.

In adaptive immunity, mucosal immunoglobulin A response directed at the carbohydrate recognition domain (CRD) of the parasite Gal/GalNAc lectin was reported to be potentially important as it was associated with protection of preschool children against amoebiasis in Bangladesh (Haque *et al*., 2001). Further researches on the dynamics of the interaction of *E. histolytica* trophozoite with its host are ongoing in trying to better understand the pathogenesis of amebiasis (Moonah *et al.*, 2013).

1.5 Treatment of Amoebisis

There are four antiparasitic drugs commonly used to combat *E. histolytica* infection which is metronidazol, Tinidazole, Paromomycin, and Diloxanide furoate. Among these, Metronidazol is commonly used to kill *E. histolytica* infection. Metronidazol is

belong to nitroimidazole group. It takes advantage of the bacteria-like enzyme known as nitroreductase in microaerophilic *E. histolytica*. The activation step of metronidazole begins through enter the cell as a prodrug by passive diffusion and electron transfer to the nitro, resulting in formation of short-lived cytotoxic nitroso free radical which includes the transfer of an electron to the nitro group of the drug that can interact with the DNA molecule. Then, activated metronidazole compound can inhibit DNA synthesis and induce DNA damage through oxidation, resulting in single- and double-strand breaks that lead to DNA degradation and cell death. Another drug that can be used to combat *E. histolytica*, known as tinidazole, also has the same mechanism of action as metronidazol (Lofmark, Edlund & Nord 2010).

Moving on, the third antiparasitic drug used to treat *E. histolytica* infection is known as paramomycin. Its mechanism of action is mainly to block the peptide synthesis at the level of ribsosome (Murray, Rosenthal & Pfaller 2013). The last drug used as part of antiparasitic therapy is diloxanide furoate, which has unknown mechanism of action against *E. histolytica*. Table 1.1 shows drug therapy for treatment of amoebiasis with their respective dosage and duration (Mandell, Douglas & Bennett 2015).

Table 1.1: Drug therapy for treatment of amoebiasis (Mandell, Douglas & Bennett 2015).

TABLE 274-2 Drug Therapy for Treatment of Amebiasis

DRUG	ADULT DOSAGE	SIDE EFFECTS
Amebic Liver Abscess*		
Metronidazole†	750 mg PO tid × 10 days	Primarily GI side effects: anorexia, nausea, vomiting, diarrhea, abdominal discomfort, or unpleasant metallic taste; disulfiram-like intolerance reaction to alcoholic beverages; neurotoxicity, including seizures, peripheral neuropathy, dizziness, confusion, irritability
or		
Tinidazole	2 g PO once daily × 5 days	Primarily GI side effects and disulfiram-like intolerance reaction to alcoholic beverages for 5 days
Followed by a luminal agent		
Paromomycin	30 mg/kg/day PO in three divided doses per day × 5–10 days	Primarily GI side effects: diarrhea, GI upset
or		
Diloxanide furoate	500 mg PO tid × 10 days	Primarily GI side effects: flatulence, nausea, vomiting Pruritus, urticaria
Amebic Colitis‡		
Tinidazole	2 g PO once daily × 5 days	Same as for amebic liver abscess
Plus a luminal agent (same as for amebic liver abscess)		
Asymptomatic Intestinal Colonization		
Treatment with luminal agent as for amebic liver abscess		

*Amebic liver abscess may necessitate antiparasitic treatment plus percutaneous or surgical drainage. Nitazoxanide may be effective therapy as well, but clinical experience is limited.
†Drug of choice for treatment of amebic liver abscess.
‡Amebic colitis may necessitate antiparasitic treatment plus surgical treatment.
GI, gastrointestinal; PO, by mouth.
Modified from Haque R, Huston CD, Hughes M, et al. Current concepts: amebiasis. N Engl J Med. *2003;348:1565-1573.*

1.6 Prevention and Control of Amoebiasis

Methods of prevention and control are much better than drugs. Elimination of the cycle of infection requires education about the routes of transmission and adequate sanitation measures, especially through chlorination and filtration. Water should be boiled and fruits and vegetables should be thoroughly cleaned before consumption (Mandell, Douglas & Bennett 2015). Unfortunately, there are no known drugs for chemoprophylaxis usage exists, but steps for prevention and control of amoebic infection are available as of yet.

1.7 Diagnosis of Amoebiasis

1.7.1 Laboratory Diagnosis

The laboratory tests used at present to diagnose amoebiasis are microscopys, antigen detection, Culture, ultrasound, biopsy serology tests and polymerase chain reaction. Intestinal amoebiasis may present as amoebic colitis or amoebic dysentery. Stool samples for three consecutive days are collected and sent for microscopy examination. Generally, stool examinations of patients suffering from amoebic liver abscess are negative. Polyvinyl alcohol (PVA) fixative or Schaudinn's fixative should be used during the stool specimen collection to preserve the fragile and rapid deteriorating trophozoites. However, direct wet mount on fresh stool samples are commonly performed in many laboratories to save cost and time, but the sensitivity is low. Therefore concentration techniques such as zinc sulphate flotation technique and formalin ether sedimentation technique should be performed on 'negative' samples to decrease the possibility of false negative result. In many cases, live trophozoites could not be detected via concentration technique, as many will deteriorate during the process (Parija *et al.*, 2014).

1.7.1.1 Stool Microscopy

The diagnosis of amoebiasis is mainly based on clinical syndrome and microscopic examination of stool samples, which posed many problems. The first problem is poor correlation between patients infected with amoeba and the development of symptomatic amoebiasis, as 90% of infected individuals present as asymptomatic carriers and amoebiasis is often clinically under-diagnosed in developing areas, unless there is history of the patients returning from tropical area (Barrett-Connor, 1971; Parija *et al.*, 2014). On the other hand, because of the morphological similarity

with the non-pathogenic strains, such as *E. dispar*, over-diagnosis and treatment were common. The second problem is the poor sensitivity of laboratory methods and low laboratory proficiency (Mukhopadhyay *et al.*, 2000; Fotedar *et al.*, 2007a). However, microscopic examination of stool samples for the protozoan morphology is still commonly practiced in many parasitology laboratories, especially in underdeveloped countries. It is difficult or even impossible to microscopically differentiate among all the human intestinal protozoa with similar morphological features, as shown in Figure 1.5 and Table 1.2 (Haque *et al.*, 1995; WHO, 1997; Tanyuksel and Petri, 2003; Haque and Petri, 2006; Fotedar *et al.*, 2007b; Liang *et al.*, 2009a).

Table 1.2 Morphologic features and pathogenicity of intestinal amoeba (Tanyuksel and Petri, 2003)

Characteristics	***E. histolytica*, *E. dispar* and *E. moshkovskii*[a]**	***E. hartmanni***	***E. coli***	***E. polecki***	***E. nana***
Trophozoites (size, nucleus, and movement)	15-20 μm; 1 nucleus; actively motile cytoplasmic protrusions, quickly finger shaped pseudopodium	8-10 μm; 1 nucleus; nonsuccessive	20-25 μm; 1 nucleus; slow movement, short and blunt pseudopodium	15-20 μm; 1 nucleus; motility resembles *E. coli*	7-9 μm; 1 nucleus, blunt and hyaline pseudopodium, slow movements
Cysts (size, nucleus)	12-15 μm; mature cyst has 4 nuclei, immature cyst has 1 or 2 nuclei	6-8 μm; mature cyst has 4 nuclei; immature cyst has 1 or 2 nuclei; 2 nucleated cysts very common	15-25 μm; mature cyst has 8 nuclei, rarely 16 or more nuclei	10-15 μm; 1 nucleus, very rarely binucleated or quadrinucleated	6-8 μm; 4 nuclei

Characteristics	***E. histolytica*, *E. dispar* and *E. moshkovskii*[a]**	***E. hartmanni***	***E. coli***	***E. polecki***	***E. nana***
Appearance of trophozoites	Stained trophozoites with fine, uniform granules of peripheral chromatin, and small central karyosome in nucleus; ingested RBC (*E. dispar* and *E. moshkovskii* are similar to *E. histolytica* trophozoites, sometimes ingested RBCs)	Nuclear structure similar to *E. histolytica*; cytoplasm finely granular; ingested bacteria	Nuclear with irregular cluster of peripheral chromatin; large, irregular, eccentric karyosome	Nucleus with minute central karyosome, with fine granules of peripheral chromatin, finely granular cytoplasm; ingested bacteria	Nucleus with large karyosome; no peripheral chromatin
Appearance of cysts	Typical nuclear structure is uniform size in having both karyosome and peripheral chromatin, chromatoidal bars with squared or rounded ends	Typical nuclear structure, chromatoidal bars with rounded or squared ends	Typical nuclear structure, sliver-shaped or irregular chromatoidals	Mononucleated; large central karyosome; chromatoid bars with pointed or angular ends, inclusion masses	Chromatin, 4 nuclei with large karyosomes and no peripheral chromatin
Pathogenicity	Only *E. histolytica* is pathogenic (*E. dispar* and *E. moshkovskii* are nonpathogenic)	Nonpathogenic	Nonpathogenic	Nonpathogenic	Nonpathogenic

[a]*E. moshkovskii* is present in free-living protozoa.

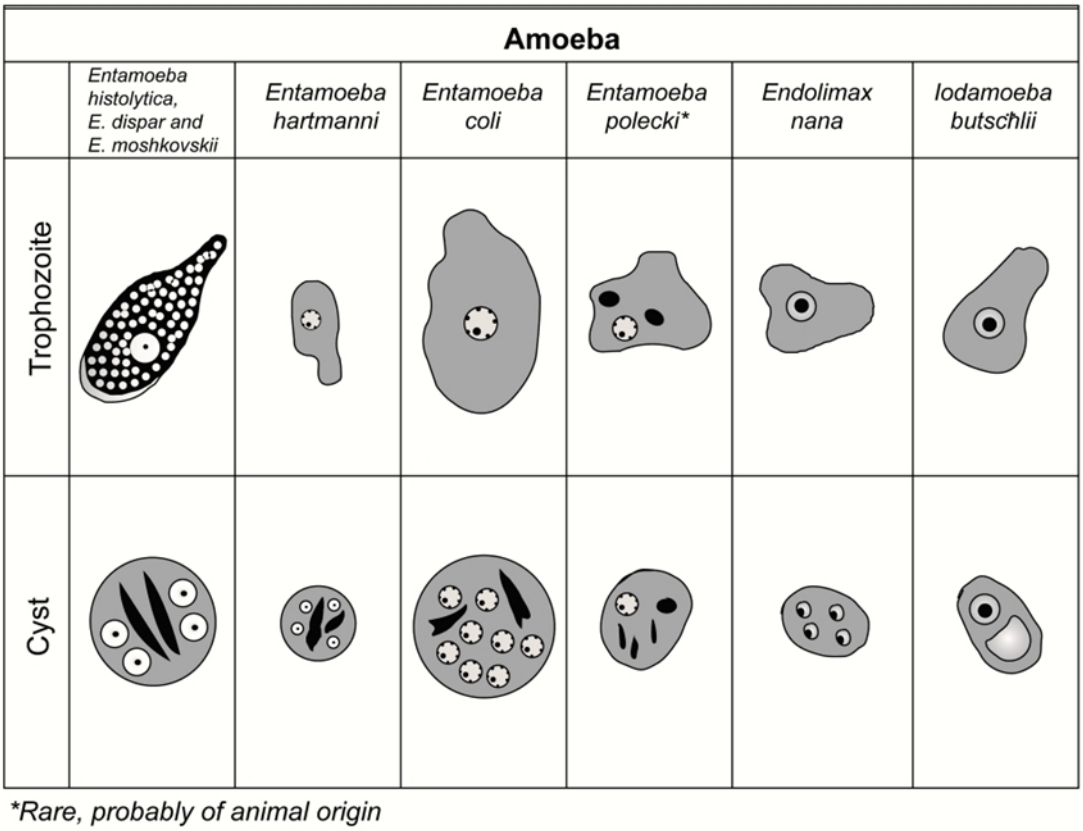


Figure 1.5 Amoebas found in stool specimens of humans (Baron, 1996)

The microscopy examination of stool samples can be performed directly or after staining. For direct stool examination, which primarily looks for stool consistency (liquid, soft or formed), blood and mucus. Liquid specimens should be examined within 30 minutes of passage; semiformed, within 1 hour of passage; and formed, within 24 hours of passage. Trophozoite and cyst can be easily identified, but the nucleus or central karyosome is difficult to see (Garcia &Shimizu, 1998, Engelkirk, & Duben-Engelkirk, 2008,). There are many stains that can be used for staining and can the internal features of trophozoite and cyst are easily visible e.g. (Lugol's iodine stain, methylene blue, Giemsa, Wright's and iodine-trichrome). However, Wheatley's trichrome staining and modified iron haematoxylin permanent stain have been suggested for routine used (Fotedar *et al*., 2007a).Wheatley's trichrome staining, trophozoites in stool samples are stained blue purple, while the background

is stained light green. In addition, this stain also displays good chromatin lining and central karyosome of the nucleus.

1.7.1.2 Stool Antigen Detection

Antigen detection test for diagnosis of intestinal amoebiasis offers several advantages. This test is able to differentiate between *E. histolytica* and *E. dispar*. The most commonly used antigen detection test is the TechLab *E. histolytica* II (TechLab, Blacksburg, VA), which is based on detection of *E. histolytica* lectin. This kit showed high sensitivity and specificity for detection of *E. histolytica* in stools samples; hence it is very useful for the diagnosis of intestinal amoebiasis. Previous studies had shown it has sensitivities of 96%, 41%-74%, and 43% when it was used to test for the presence of Gal/GalNAc lectin in the serum, liver abscess pus, and stool specimens collected at the time of diagnosis of amebic liver abscess. However, the commercially available tests are too costly for most developing countries, as compared to conventional microscopic methods (Roy *et al*., 2005; Haque *et al*., 2000).

1.7.1.3 Stool Culture

There are three types of culture techniques for cultivation of *E. histolytica* namely xenic, monoxenic and axenic culture methods. In xenic cultivation, *E. histolytica* can be detected by using media such as Locke-egg medium, Robinson's medium or TYSGM-9 medium. In either monoxenic or axenic method, the media that can be used are TYI-S-33, YI-S and LYI-S-2 media. Culture and isolation of *E. histolytica* can be done using stool specimens, rectal biopsy specimens and liver abscess aspirates. However, the success rate is only between 50% and 70% with a significant

false-negative rate. Besides, such culture technique for *E. histolytica* from stool is only available in few research laboratories worldwide. Furthermore, culturing of the parasite is a difficult, expensive and labour-intensive technique, so nowadays this method is no longer a routine practice in diagnostic laboratories (Clark & Diamond, 2002).

1.7.1.4 Molecular Diagnosis

PCR assay can differentially detect specific genes of *Entamoeba* species in stool specimens and liver pus aspirates. This method is very sensitive as it is reported to detect as little as 10 pg of *E. histolytica* and *E. moshkovskii* DNA, and 20 pg of *E. dispar* DNA using conventional PCR test (Hamzah *et al.*, 2006). DNA extraction step is crucial prior to PCR test because the presence of PCR inhibitors in faeces (*e.g.* heme, bilirubins, and bile salts) causes false-negative results. Thus, optimisation of faecal DNA extraction step is required in order to remove the unwanted inhibitors results (Holland *et al.*, 2000).

On the other hand, PCR assay is able to detect amoebic DNA in liver pus aspirates with overall sensitivity rate of ~80%. Besides, detection of amoebic DNA *via* PCR test can be adopted on other clinical samples such as blood, urine and serum, too. (Ahmad *et al.*, 2007; Parija and Khairnar, 2007; Othman *et al.*, 2010). The study showed that saliva and urine were the diagnostic tools for ALA, with the sensitivity of 97% (Haque *et al.*, 2010).

In summary, PCR best tests and assays for the diagnosis of *E. histolytica* Abscess are as follow:

- PCR is known with as highly sensitive and specific in detection and differentiating *E. histolytica* and *E. dispar*, as compared to ELISA (Mirelman *et al.*, 1997).
- Various PCR primer pairs have been developed to effectively distinguish pathogenic *E. histolytica* from non-pathogenic *E. dispar* and *E. moshkovskii*, which include small-subunit RNA nucleotide sequence, tDNA associated short tandem repeats (STRs), DNA highly repetitive sequences, extra-chromosomal circular DNA, hemolysin gene (*HLY6*) LSU rRNA, cysteine proteases and lectin gene (Zindrou *et al.*, 2001; Fotedar *et al.*, 2007b; Fotedar *et al.*, 2007c; Paul *et al.*, 2007).
- One of the disadvantages of using antibody assay is that it cannot differentiate current from past infections; PCR diagnostic method has the potential to overcome this limitation by detecting the presence of parasites in the clinical samples (Rochelle *et al.*, 1997).
- It has greater sensitivity, rapid analysis, simultaneous detection of multiple pathogens and the ability to differentiate between species by using species-specific primer pairs.

1.7.1.5 Serology

1.7.1.5.1 CSA Antigen

The axenic culture of *E. histolytica* trophozoites using TYI-S-33 medium, established in 1961 by Louis Diamond and his team members has led to the rapid pace of scientific discovery on this parasite, as many *in vivo* and *in vitro* studies can

be conducted by means of pure culture. Besides, pure antigen can be produced directly from the culture for diagnosis use, as well as the study of sero prevalence (Wong *et al.*, 2017).

1.7.1.5.2 ESA Antigen

Excretory and secretory (ES) products contain virulence factors like amoebapores, cysteine proteases, collagenases, glycosidases and other proteases that has been hypothesized to contribute to the pathogenesis of *E. histolytica*. During active infection, *E. histolytica* trophozoites secrete and/or excrete products into the host environment (Bhattacharya *et al.*, 1998). These secreted products were suggested to be important for the establishment and maintenance of the parasite in its host (Munoz *et al.*, 1982; Gitler *et al.*, 1984; Guerrero-Manriquez *et al.*, 1998; Debnath *et al.*, 2005; Moncada *et al.*, 2005). As compared to conventionally used crude soluble antigen in an ELISA format, ESA was shown to increase the detection rate in patient suffering from acute amoebic dysentery and asymptomatic cyst passers groups, as well as be equally sensitive for detection of ALA (Pal *et al*., 1996).

1.7.1.5.3 Amoebic Lectin Antigen

TechLab *E. histolytica* II (TechLab, Blacksburg, VA) has also been investigated for use in detection of *E. histolytica* circulatory lectin antigen in stool, saliva, serum and aspirated liver pus from patients (Haque &Petri, 2006). The detection of lectin antigen in the aspirated liver pus samples was shown to be 100%, provided the specimen was taken prior to any treatment. Detection of lectin antigen in the saliva was shown to be 70% in sensitivity; while, the sensitivity for detection of lectin

antigen in the human serum samples with ALA was reported to be 75% for the late stage of infection and 100% for the first three days after onset of the symptoms patients. There is study showing sensitivity for detection of lectin antigen in saliva samples of ALA patients was only 22%, while higher (66%) in cases of amoebic colitis (Abd-Alla *et al.*, 2000). A summary of the sensitivities and specificities of antigen detection tests for diagnosis of amoebiasis is shown in Table 1.3 (Tanyuksel & Petri, 2003; Fotedar *et al.*, 2007a).

Table 1.3 Commercial assays used to identify *E. histolytica*

Assay	Sensitivity (%)	Specificity (%)	Manufacturer
Antigen detection			
TechLab *E. histolytica* II	100[a]	~95[a]	TechLab, Blacksburg, Va.
TechLab *Entamoeba* test	80[b]–95[c]	99[b]–93[c]	TechLab, Blacksburg, Va.
ProSpecT *Entamoeba histolytica* microplate assay	90.3[d]	97.7[d]	Alexon-Trend Inc., Ramsey, Minn.
Entamoeba CELISA-*PATH*	KP[o] (94)	KP (100)	Cellabs Pty Ltd., Brookvale, Australia
Entamoeba-CELISA-Screen	KP (87.7)	KP (98.3)	Cellabs Pty Ltd., Brookvale, Australia
Wampole *E. histolytica* Test	KP (94.7)[e]	KP	Wampole Laboratories, Cranbury, N.J.
Merlin Optimun S ELISA	100[f]		Merlin Diagnostika, Bernheim-Hersel, Germany
Triage parasite panel	68.3[g]	100[g]	BIOSITE Diagnostics, San Diego, Calif.
	83.3[h]	100[h]	
	96[i]	99.1[i]	
Amibiase Ag EIA	NP[p]	NP	Biotrin Int., Dublin, Ireland
Antibody detection			
IHA Cellognost Amoebiasis	72.2[j]–100[k]	99.1[j]–90.9[k]	Behring Diagnostics, Marburg, Germany
Amibiasis Serology Microwell EIA	92.5[l]	91.3[l]	LMD Laboratories Inc., Carlsbad Calif.
BLA-Bichrolatex-Amibe	98.3[m]	96.1[m]	Fumouze Diagnostics, Levallois-Perret, France
IHA	93.4[m]	97.5[m]	Fumouze Diagnostics, Levallois-Perret, France
The Melotest Amoebiasis EIA	NA[n]	NA[n]	Melotec, S.A., Barcelona, Spain

[a]Compared to culture and isoenzyme analysis ,[b]Compared to culture,[c]Compared to zymodeme analysis

[d]Compared to microscopy ,[e]Correlation to zymodeme analysis.

[f]Correlation of TechLab *E. histolytica* for detection of *E. histolytica*, but not *E. dispar*

[g]Compared to ProSpecT ELISA ,[h]Compared to microscopy ,[i]Compared to O&P and permanent stains

[j]Reference 91,[k]Reference 161 (with use of *E. histolytica* antigen detection as the reference standard).

[l]Reference 204,[m]Reference 180,[n]Reference 182. NA, not available,[o]KP, kit prospectus

[p]NP, not published data.

1.7.1.6 Diagnosis of Extraintestinal Amoebiasis

The examination for extraintestinal amoebiasis is often started when patient experiences the symptoms of fever, nausea, hepatomegaly and tenderness in the right upper quadrant. Radiological imaging is performed to investigate the presence and size of the abscess. Depending on the physical condition of the patient and size of the abscess; The physician may aspirate the abscess for microscopy examination to check for the presence of live trophozoites, and also to rule out the possibility of liver necrosis (Salles *et al.*, 2003). For extraintestinal amoebiasis cases, the symptoms of intestinal amoebiasis may not be present, and trophozoites and cysts are rarely found

in the stool samples. Therefore, many physicians often treat the patient based on the combination of clinical manifestations, serological test and radiological imaging (Baron, 1996). In addition to that, abdominal imaging techniques such as ultrasound, computer tomography, and magnetic resonance are useful adjuncts for diagnosis of intestinal and extraintestinal disease.

1.8 Rationale of the Study

Orang Asli who dwelled in remote settlements with substandard sanitation and poor supply of safe water are high risk group for amoebiasis (Ngui *et al*., 2012). For control and prevention measures, continuous surveillance of the prevalence and distribution of anti-amoebic antibody in Orang Asli settlements are important (Alum *et al*., 2010). However, mass screening by commercial kits is impractical due to the high cost. Cheaper but valid in-house assay should be used instead to attain cost-effective and sustainable outcome (Tan *et al*., 2013). Customized CSA-ELISA is widely used for detection of anti-amoebic antibody, but the undefined antigenic properties and high proteases content have reduced assay reproducibility (Flores *et al*., 2016). Otherwise, customized ELISA using rLectin protein with defined antigenic properties and relatively ease to produce might be more suitable for mass screening purpose (Stanley *et al*., 1998).

1.9 Objective of the study

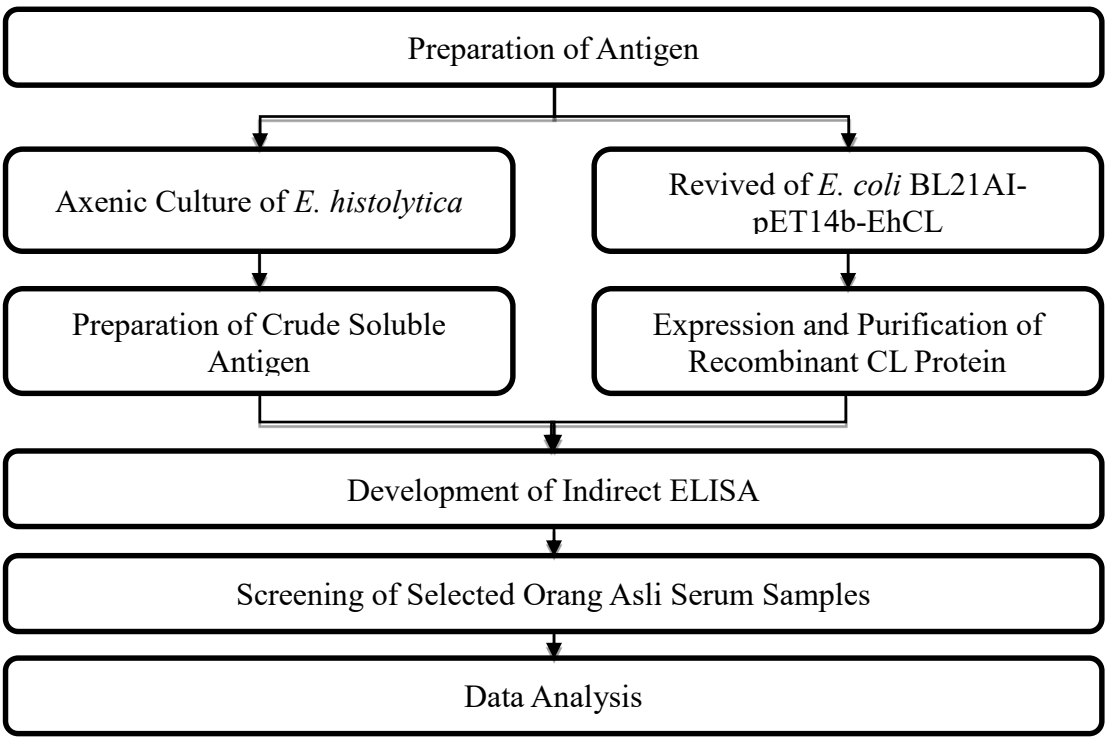
The general objective of the study is to compare the efficiencies customized CSA- and rLectin-ELISAs for detection of anti-amoebic antibody in selected Orang Asli serum samples defined by commercial IHA.

The specific objectives include:

1. To produce *E. histolytica* CSA and rLectin protein for detection
2. To develop and optimize the customized CSA- and rLectin-ELISAs
3. To compare the efficiencies of both customized ELISAs

1.10 Overview of the Study

The methodology of the study is as shown below.



CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1 *Entamoeba histolytica* culture

*E. histolytica HM-1:*IMSS was first isolated in 1967 from an adult male in Mexico City who was suffering from rectal ulcer. It was then gradually adapted to axenic culture in Hospital de Especialidades, Instituto Mexicano del Seguro Social, Mexico City. In this study, this axenic strain was a kind contribution from Dr. Alfonso Olivos-Garcia from Departamento de Medicina Experimental, Facultad de Medicina, Universidad Nacional Autónoma de México.

2.1.2 *Escherichia coli* Strain

E. coli BL21AI (Invitrogen, USA) carrying pET14b-rLectin was used for production of recombinant lectin protein.

2.1.3 Serum Samples

Archived Orang Asli serum samples used in the present study were previously analysed by indirect haemagglutination assay (Dade Behring Marburg GmbH, Marburg, Germany). The use of these serum samples was previously approved by Malaysian Ministry of Aborigine Affairs (Appendix 1). Serum samples used for evaluation of CSA- and rLectine-ELISAs were as follow:

Group I: IHA seropositive serum samples: Anti-amoebic antibody titer >128 (N=33)

Group II: IHA seronegative serum samples: Negative by IHA screening (N=30)

2.1.4 List of Chemicals

Chemicals used in this study are listed in Table 2.1.

2.1.5 List of Kits and Consumables

Kits and consumables used in this study are listed in Table 2.2.

2.1.6 List of Equipment

Equipment used in this study is listed in Table 2.3.

2.1.7 List of Buffers and Reagents

Buffers and reagents used in the present study are listed in Appendix 2.