EFFICACY OF INDIRECT ELISA USING CRUDE SOLUBLE *Entamoeba histolytica* ANTIGEN FOR THE DIAGNOSIS OF AMOEBIC LIVER ABSCESS

by

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CERTIFICATE

This is to certify that the dissertation entitle "Efficacy of Indirect ELISA Using Crude Soluble *Entamoeba histolytica* Antigen for the Diagnosis of Amoebic Liver Abscess" is the bonafide record of the research work done by:

Khairul Nissa Bt Saidun

During the period from July 2009 to October 2009 under my supervision.

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LIST OF ABBREVIATIONS

ALA	Amoebic liver abscess
BSA	Bovine Serum Albumin
COV	Cut Off Value
CSA	Crude Soluble Antigen
CT	Computed tomography
EDTA	Ethylenediaminetetraaacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
FAC	Ferric Ammonium Citrate
Gal/GalNAc	Galactose/N-acetylgalactosamine
HRP	Horseradish Peroxidase
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHA	Indirect Haemagglutination Assay
NPV	Negative Predictive Value
OD	Optical Density
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PPV	Positive Predictive Value
r.e.p.	Repetition
TMB	3,3',5,5' Tetrametylbenzidine
TYI-S-33	Trypticase-Yeast-Iron Serum-33

v/v	volume to volume
w/v	weight to volume

ABSTRAK

Amebiasis merupakan penyakit yang disebabkan jangkitan protozoa parasit, iaitu *Entamoeba histolytica*. Terdapat dua jenis penyakit amebiasis, iaitu amebiasis dalam usus, dan di luar usus. Dijangkakan 10% populasi dunia menghidap jangkitan ini. Penanahan hati amebik (ALA) adalah kesan komplikasi jangkitan amebiasis yang telah menular keluar dari usus. Antara penyakit yang disebabkan oleh jangkitan parasit, penanahan hati amebik merupakan yang jangkitan kedua berbahaya selepas malaria dengan kadar kematian yang tinggi. Walaupun penyakit ini boleh diubati, namun ianya sudah terlewat apabila ditemui memandangkan hati telah mengalami kerosakan yang teruk. Dalam kajian ini, keberkesanan antigen kasar terlarut *E. histolytica* telah diuji menggunakan ujian ELISA Tak Langsung (Indirect Enzyme-Linked Immunosorbent Assay). Protein yang telah dihasilkan ditentukan kepekatannya, iaitu 5839.1 µg/ml. Asai tersebut telah dioptimumkan sebelum diuji keberkesanan protein. Kepekatan protein yang optimum ialah 20 µg/mL, manakala pencairan optimum bagi antibodi primer dan sekunder ialah masing-masing 1:25 dan 1:500. Sampel serum yang digunakan dalam kajian ini terlebih dahulu diuji bagi mengesan antibodi penanahan hati amebik menggunakan asai perlegatan-darah tidak langsung (Indirect Haemeagglutination Assay). ELISA tak langsung tersebut telah dijalankan ke atas 30 sampel serum yang negatif (individu sihat) bagi memperoleh ("Cut-Off Value") iaitu 0.5677. Nilai ketumpatan optikal yang kurang daripada 0.5677 dianggap negatif. Peratus kepekaan dan spesifisiti bagi asai ini ialah masing-masing 81.3% dan 96.7%.

ABSTRACT

Amoebiasis is an infection of a parasitic protozoan, *Entamoeba histolytica*. There are two types of amoebiasis, intestinal amoebiasis and extraintestinal amoebiasis. Ten percent of world population was infected by amoebiasis, and high mortality rate was recorded due to amoebic liver abscess (ALA) second after malaria. Although it is treatable, it is usually too late when detected. Many clinical tests and researches are underway to improve the diagnosis of amoebic liver abscess. In this study, the efficiency of Indirect ELISA using crude soluble antigen of *E. histolytica* for the diagnosis of ALA was investigated. The serum samples used in this study were tested with Indirect Haemaaglutination Assay (IHA) for ALA antibodies. The crude soluble protein was produced from whole cell lysate by sonication. The concentration of the protein measured by Bradford assay was 5839.1 µg/ml. The assay was optimized prior testing the efficacy. The optimum concentration of the antigen was 20 µg/mL, 1:25 and 1:500 were the optimum dilutions of primary and secondary antibodies, respectively. Cut-Off Value (COV) that has been determined from 30 IHA negative ALA serum samples readings using Indirect ELISA was 0.5677. Optical density (OD) reading of the serum sample tested with Indirect ELISA that less than this point was regarded as negative. The sensitivity and specificity of the assay using crude soluble antigen were 81.3% and 96.7%., respectively.

CHAPTER 1

INTRODUCTION

1.0 Introduction

Amoebiasis has been defined as an infection with parasitic protozoan, *Entamoeba histolytica* (*E. histolytica*). According to World Health Organization (WHO, 1997), over 50 million people have been infected by amoebiasis, and more than 100,000 individuals died due to invasive amoebiasis annually. Among the parasitic diseases, amoebiasis is the second leading cause of death after malaria (Laughlin and Temesvari, 2005). This intestinal amoeba was first discovered in 1875, by Fedor Losch, from a Russian patient with dysentery stool (Fedor Losch, 1975). According to an article by Petri *et al.,* (1999), stated that invasive amoebiasis was acquired through the distruption and destruction of the intestinal epithelial tissues, disseminating circulation system and resistance to host's immune defenses. *E. histolytica* is found worldwide with higher prevalence in tropic and subtropic climates and significantly in overcrowding regions, reduced clean water supply, poor socioeconomic and sanitary conditions (Stanley, 2003; Tanyuksel and Petri, 2003).

1.1 *Entamoeba histolytica*

E. histolytica is an anaerobic parasitic protozoan known to pose threat to human and infection by this amoeba results in amoebiasis. Another morphologically identical but non-pathogenic species is *Entamoeba dispar*. However, only *E. histolytica* elicits serious complication. There are 4 distinct stages in its life cycle; trophozoite, precyst, cyst, and metacyst, only trophozoite and cyst possess morphological characteristics that have diagnostic value. The genetic characterisation of *E. histolytica* are useful in differentiation of pathogenic amoeba from other non-pathogenic species. Trophozoite has an amoeboid appearance, 15 to 30 μm in diameter and single nucleus with centrally located karyosome. It is the active form while cyst is the non-motile form. Trophozoite stained with trichrome stain gives clear nuclear structure. Nuclear membrane is visible as a delicate but distinct line; peripheral chromatin granules are fine and uniformally arranged on the inner surface of the nuclear membrane; and karyosome is small and centrally located (Ortega, 2006).

Prior encysting, trophozoites round up, cease ingesting food and secrete a cyst wall, thus becoming a precyst and then an immature and mature cyst. The immature cyst is spherical in shape and consists of 1-2 nucleus or nuclei but the number of the nucleus increase as the cysts mature, as many as 4 nuclei. Besides nuclei, there are two other inclusions present in the cyst which are the glycogen vacuole and chromatoid bodies. Both the glycogen and chromatoid bars become smaller and smaller as the cyst ages. Therefore, sometimes they cannot be seen in the mature cysts.

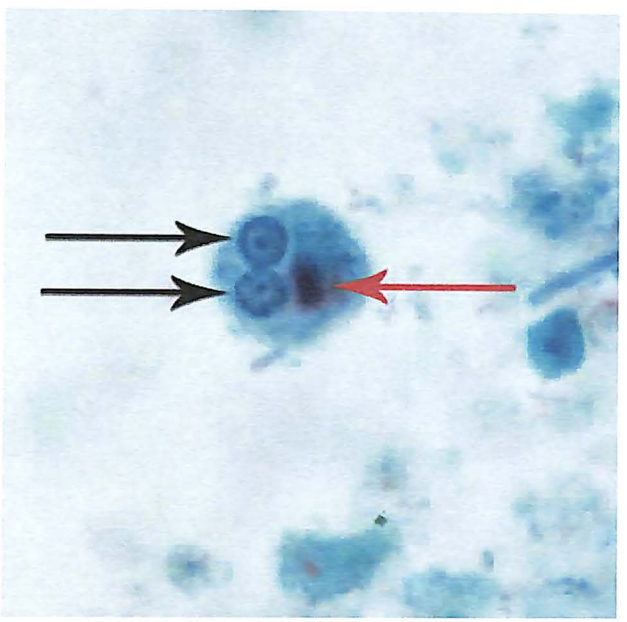

Figure 1.1: *E. histolytica* cyst stained with trichrome stain. Cyst contain two nuclei (black arrow) and chromatoid body (red arrow)

(www.dpd.cdc.gov/dpdx/HTML/ImageLibrary/Amoebiasis)

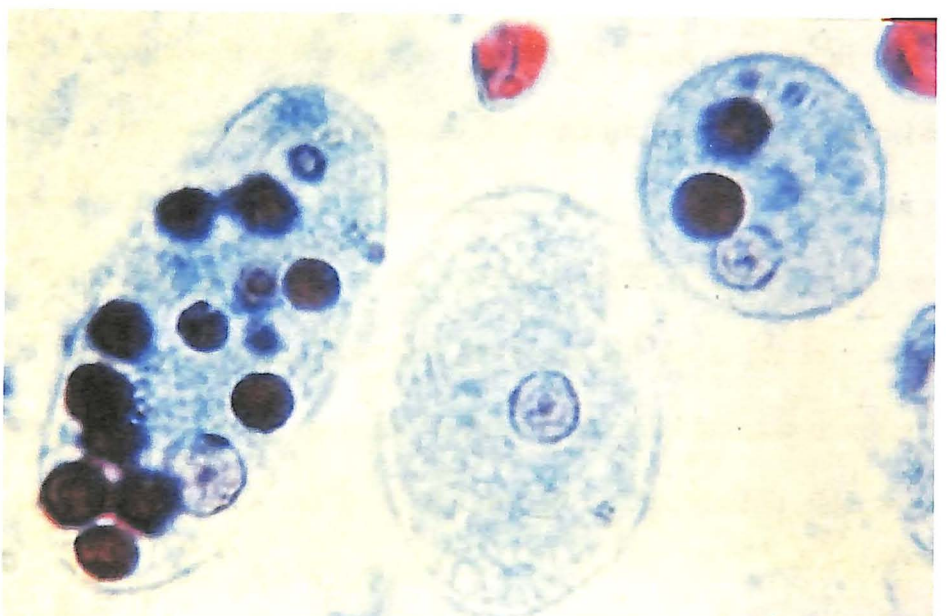

Figure 1.2: Trophozoites of *E. histolytica* stained with trichrome stain. The ingested erythrocytes appear as dark inclusions and centrally located karyosome with peripheral chromatin in the nuclei.

(www.dpd.cdc.gov/dpdx/HTML/ImageLibrary/Amoebiasis)

1.2 *E. histolytica* Life Cycle and Transmission

Life cycle of *E. histolytica* does not require intermediate host, but it consists of an infective cyst and invasive trophozoite forms. Ingestion of cysts that are resistance to gastric acid, survive its way through stomach. Due to acidic condition, the cysts will excyst in ileum releasing active trophozoites. Trophozoites present in large intestine divided by binary fission into metacyst and further divide into four and finally into eight amoebas. As they reach down the large bowel, under some condition the trophozoites will encyst and release into faeces. Thick outer cell walls of the cysts favor their survival in moist environment from several weeks to months. However, the cysts will die either in moderate heat or freezing temperature. Although the trophozoites are normally found in diarrheal stool, they are easily destroyed outside the host, and barely survive gastric acid when ingested (Tanyuksel and Petri, 2003; AWWA, 2006).

Transmissions of *E. histolytica* are commonly occur through oral ingestion and consumption of contaminated water sources. The prevalence of the pathogen is largely influenced by the quality and quantity of clean water available for drinking and washing. A study being done in high prevalence area of amebic liver abscess (ALA) in Central Vietnam shows that poor sanitation, limitation to clean water supply and consumption of contaminated water increase the risk of ALA (Blessmann *et al.*, 2002).

Persons at greater risk for amoebiasis includes immigrants from endemic areas, travelers to tropics, homosexual men, immunosuppressed individual, and mentally retarded people living in an institution (Petri and Singh, 1999).

A study update of *E. histolytica*, shows that the disease complications varies geographically, as such amoebic colitis is prevalence in Egypt than the high rates of ALA in Central Vietnam and highest in South Africa (Stauffer and Ravdin, 2003).

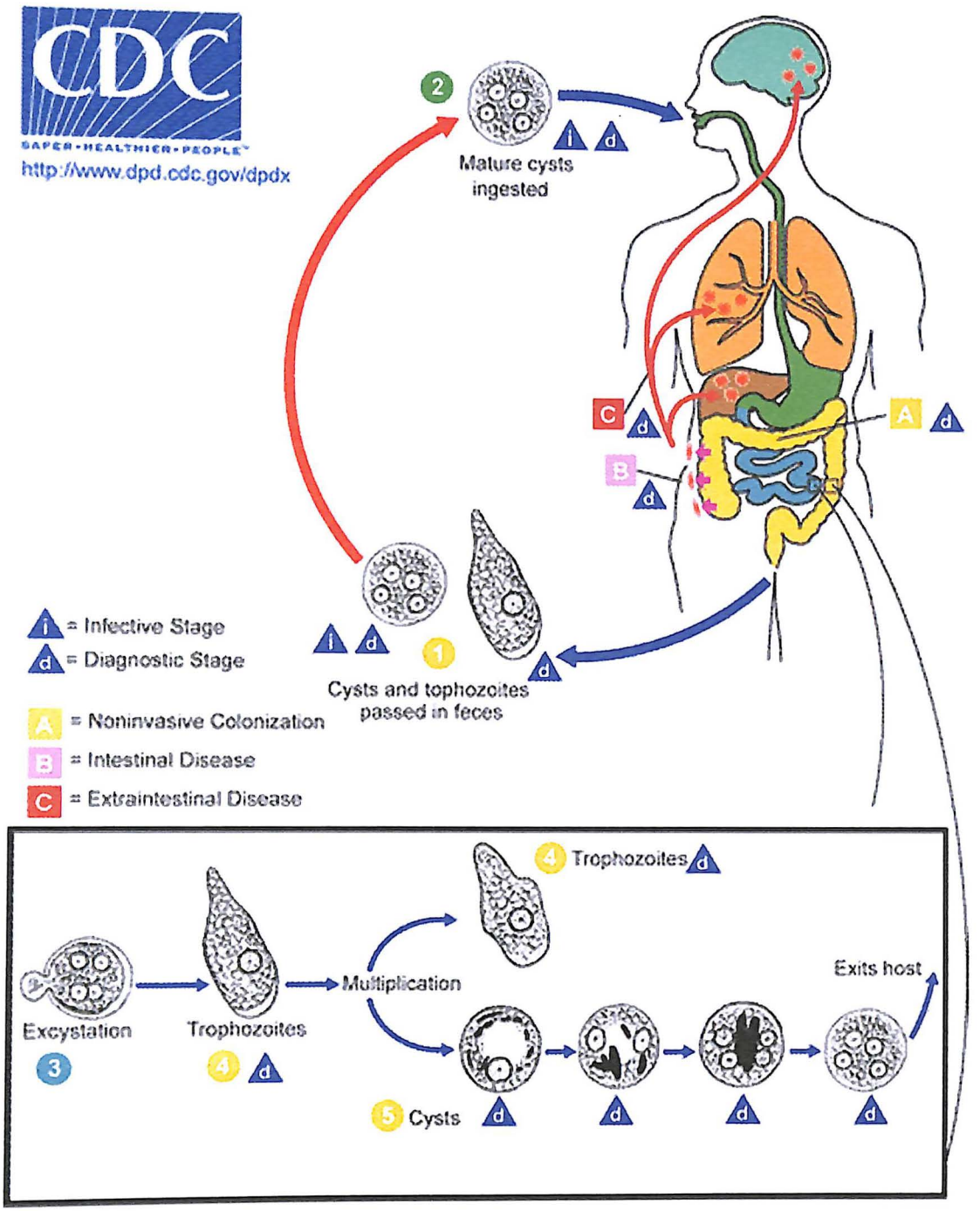

Figure 1.3: Life cycle of *E. histolytica*

(www.dpd.cdc.gov/dpdx/HTML/Image_Library.htm)

1.3 Pathogenecity of Amoebic Liver Abscess (ALA)

Fedor Losch's patient was the first known to have died from amoebiasis. Autopsy revealed intestine was edematous, hyperemic together with interspersed microhaemorrhages. Thickening and mass ulceration of large intestine was due to inflammatory infiltrate (Fedor, 1975).

According to Ravdin (1989), there are four steps that must take place in order for the amoeba to become invasive. First, there is binding between trophozoites either to intestinal epithelium or colonic mucus, or both. Second, the trophozoites invade the epithelium and diminish its barrier. Third, the trophozoites are able to induce lysis of the epithelial cells. Fourth, is the resistancy of the amoeba against host immune response. Whereas, other factors that have contribute to tissue invasion are adhesin, ameobapores, proteases, various types of proteolytic enzymes and amebic-membrane associated enzymes of *E. histolytica* (Tanyuksel and Petri, 2003)

However, some aspect of pathogenesis is still controversial. The invasiveness of amoeba varies, depending on host immune system and virulency of amoeba strain. Role of genetic and immunoenzyme profile, types of proteolytic enzymes secreted, and ability to resist host complement-mediated lysis contribute to aggressiveness (Salles *et al.,* 2007).

An amebic cell surface protein, a type of adhesion, Galactose/N-acetylgalactosamine (Gal/GalNAc) lectin plays a central role for the tissue invasion has been characterized in greater details. The lectin facilitates in adherence and cytolysis, and suspected to have intracellular signaling properties. Another major factor of virulence is cysteine proteinases which able to degrade elements of cellular matrix and interfere complement pathway and

humoral immune response (Tarleton and Petri, 2003). Petri *et al.,* (2002) described that throphozoite enable rearrangement of its cytoskeletons when lectin bind to host, changing the activity of the lectin and adapt to the host immune defense, results in increase the risk of invasion. (Figure 1.4)

The right lobes are more likely to get the infection as it receives four times more of venous drainage compared to left lobe. Through cell adhesion, trophozoites lyse the neutrophil and polymorph cells subsequently release mediators contribute to death of hepatocytes, forming cellular necrosis and pose damage to distant hepatic cells. The necrotic contents of the liver abscess are described as 'anchovy sauce' or 'chocolate paste'. The extensive tissue necrosis results in hepatic damage (Pritt *et al.,* 2008).

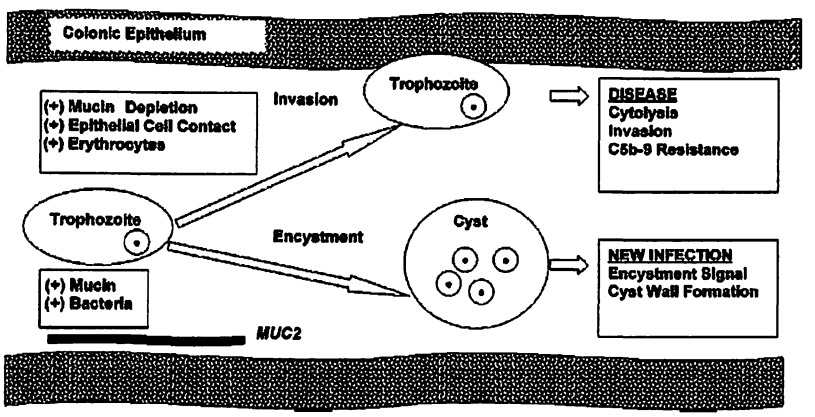

Figure 1.4 Mechanism of Gal/GalNAc; Presence of colonic mucin glycoproteins or bacteria favor the cyst formation. While, exudated red cell and depletion of the mucins favor parasite contact to epithelial cell initiate the invasion. The processes are mediated by Gal/GalNAc lectin (Petri et al, 2002).

1.4 Treatment

According to WHO (1997), a definite detection of *E. histolytica* from non-pathogenic entamoeba is crucial in suspected amoebiasis case and should be treated appropriately. Paromycin and diloxamide furoate drugs are available for treating intestinal amoebiasis. Paromycin able to retain in the bowel without being absorbed is effective in eradicating the luminal cysts thus preventing tissue invasion. The mainstray of therapy is metronidazole. Highly lethal to the trophozoite, rapid intestinal absorption, excellent bioavailability in tissues and good penetration in abscess making metronidazole as the drug of choice. Oral administration of metronidazole is preferable with 750 mg dose thrice a day. The duration of therapy is 5 to 10 days and dependent on the severity of infection. In such severe case of ALA, patient may receive 500 mg metronidazole intravenously followed by luminal amebicide therapy (Salles *et al.*, 2003).

1.5 Differential Diagnosis

The differential diagnosis of amoebic liver abscess can be problematic as detection of amoeba in stool is less sensitive and unspecific. Whereas, antibodies detection in serum may gives false result. However, diagnosis of *E. histolytica* in most endemic regions still relied on the microscopic examination of stool. The sensitivity of the technique was found to be 10% to 60% and may give false-positive due to misidentification of macrophages and non-pathogenic amoeba (Nazemalhosseini *et al.,* 2008). Furthermore, it is difficult, time consuming, expensive and tedious. Positive finding of the cyst doesn't differentiate between intestinal and extraintestinal infection, also the stool fail to demonstrate the presence of amoeba in ALA infection (Walsh, 1986).

Serology method has the advantage over stool examination as it can show presence of products due to the interaction between the *E. histolytica* and host immune respones. Enzyme-Linked Immunosorbent Assay (ELISA) is a popular method of diagnosis since the specificity and sensitivity are 94.8% and 97.9% (Hira *et al.,* 2001).

In an assessment of antibody detection using ELISA against *E. histolytica* crude soluble extract and three fractions of amoebic antigens preparations namely FI, FII, and FIII each fraction with different molecular weight from FI to FII the molecular weights are descending shows that the crude amebic antigen gives 93.3% and 70.0% positive for ALA and amebic dysentery (Arianpour and Mahopatra, 2003).

Indirect Haemagglutination Assay (IHA) is another method employed for the diagnosis especially in immunocompromised patients. In a study of amoebiasis in HIV-infected patients shows that high specificity using IHA. Although it is easy to perform, its lower