DETECTION OF ANTI-PYRUVATE: FERREDOXIN OXIDOREDUCTASE (PFOR) ANTIBODY AGAINST *ENTAMOEBA HISTOLYTICA* IN SERA OF AMOEBIASIS PATIENTS BY USING WESTERN BLOTTING

KANLAYA BOONPEN

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Kanlaya Boonpen

Miss Kanlaya Boonpen
Candidate

Nitaya Thammapalerd

Assoc.Prof.Nitaya Thammapalerd
M.Sc. (Biochemistry)
Major-Advisor

Paisarn Sithigorngul

Assoc.Prof.Paisarn Sithigorngul
Ph.D. (Zoology)
Co-Advisor

Siwaporn Longyant

Dr. Siwaporn Longyant
Ph.D. (Marine Science)
Co-Advisor

Thareerat Kalambaheti

Assist.Prof.Thareerat Kalambaheti.
Ph.D.(Microbiology)
Co-Advisor

Daroon Kotimanusvanij

Assist.Prof. Daroon Kotimanusvanij
M.Sc. (Tropical Medicine)
Co-Advisor

Rassmidara Hoonsawat

Assoc.Prof.Rassmidara Hoonsawat
Ph.D.
Dean
Faculty of Graduate Studies

Somjai Leemingsawat

Assoc.Prof.Somjai Leemingsawat,
Ph.D.
Chair
Master of Science Programme in
Tropical Medicine
Faculty of Tropical Medicine

Thesis

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For the degree of Master of Science (Tropical Medicine)

on

October 18, 2004

Kanlaya Boonpen

Miss Kanlaya Boonpen
Candidate

Thareerat Kalambaheti

Assist.Prof. Thareerat Kalambaheti
Ph.D. (Microbiology)
Member

Nitaya Thanumpalerd

Assoc.Prof.Nitaya Thanumpalerd
M.Sc. (Biochemistry)
Chair

Daroon Kotimanusvanij

Assist.Prof.Daroon Kotimanusvanij
M.Sc. (Tropical Medicine)
Member

Paisarn Sithigorngul

Assoc.Prof.Paisarn Sithigorngul
Ph.D. (Zoology)
Member

Wilai Saksirisampant

Assoc.Prof. Wilai Saksirisampant
M.Sc. (Microbiology)
Member

Siwaporn Longyant

Dr.Siwaporn Longyant
Ph.D. (Marine Science)
Member

Rassmidara Hoonsawat

Assoc.Prof.Rassmidara Hoonsawat
Ph.D.
Dean
Faculty of Graduate Studies
Mahidol University

P. Singhasivanon

Assoc.Prof. Pratap Singhasivanon
M.B.B.S.,D.T.M.&H.(Bangkok),
M.P.H., Dr.P.H.(Epidemiology)
Dean
Faculty of Tropical Medicine
Mahidol University

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KANLAYA BOONPEN 4437532 TMTM/M

M.Sc. (TROPICAL MEDICINE)

THESIS ADVISORS: NITAYA THAMMAPALERD, M.Sc.(BIOCHEMISTRY), PAISARN SITHIGORNGUL, Ph.D.(ZOOLOGY), SIWAPORN LONGYANT, Ph.D. (MURINE SCIENCE), THAREERAT KALAMBAHETI, Ph.D.(MICROBIOLOGY), DAROON KOTIMANUSVANIJ M.Sc.(TROPICAL MEDICINE)

ABSTRACT

Western blotting was developed in this study for diagnosing amoebiasis by detecting specific anti-amoebic PFOR antibody in sera of amoebiasis patients by comparison with the positive control, which was a specific 144 kDa band of immune complex between Eh208C2-2 MAb and its corresponding PFOR antigen of *E. histolytica*. 70 sera were divided into 3 groups; Group I sera were divided into 30 Group IA sera from invasive amoebiasis whose stools were positive for *E. histolytica* by microscopy and sero-positive by IHA or IEP tests and 20 Group IB sera from asymptomatic amoebiasis whose stools were positive for *E. histolytica*/*E.dispar* by microscopy; 10 Group II sera whose stools were negative for *E. histolytica* but positive for other intestinal parasites, i.e. *Entamoeba coli, Endolimax nana* and *Blastocystis hominis*; 10 Group III uninfected controls whose stools were free from any intestinal parasites. All 30 sera in group IA recognized 144 kDa which correlated well with the PFOR antigen band that reacted specifically with positive control mouse Eh208C2-2 MAb, whereas 7 of 20 sera in group IB recognized 144 kDa. All 10 group II sera did not recognized 144 kDa, of which 2 sera infected with *E. nana* were negative and 1 of 4 sera infected either with *B. hominis*, or with *E. coli* recognized 115, 83, 59 kDa or only 115 kDa, respectively, whereas all 10 group III sera were negative. When microscopy methods or serological tests were used as the gold standard, Western blotting of group IA revealed specificity and efficacy of 100%, while group IB revealed sensitivity and efficacy of 35% and 56.6%, respectively. All 50 group I sera provided 100% specificity without any cross-reactivity with other parasites. Therefore, Western blotting represents a simple, high-sensitivity, and efficacious diagnostic test for invasive amoebiasis. Application of this test in patients with amoebic liver abscess should be of diagnostic value.

KEY WORDS : ANTI-PFOR ANTIBODY, AMOEBIASIS, *E. histolytica* , SDS-PAGE, WESTERN BLOTTING

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การตรวจหาแอนติบอดีที่จำเพาะต่อแอนติเจน EhPFOR ในเซรุ่มผู้ป่วยที่ติดเชื้อบิดอะมีบา โดยวิธี WESTERN BLOTTING (DETECTION OF ANTI-PYRUVATE: FERREDOXIN OXIDOREDUCTASE (PFOR) ANTIBODY AGAINST *ENTAMOEBA HISTOLYTICA* IN SERA OF AMOEBIASIS PATIENTS BY USING WESTERN BLOTTING)

กัลยา บุญเพ็ญ 4437532 TMTM/M

วท.ม. (อายุรศาสตร์เขตร้อน)

คณะกรรมการควบคุมวิทยานิพนธ์: นิตยา ธรรมพาเลิศ วท.ม. (ชีวเคมี), ไพศาล สิทธิกรกุล วท.ด. (สัตววิทยา), ศิวาพร ลงยันต์ วท.ด. (วิทยาศาสตร์ทางทะเล), ธารีรัตน์ กะลัมพะเหติ วท.ด. (จุลชีววิทยา), ดรุณ โกฏิมนัสวนิชย์ วท.ม. (อายุรศาสตร์เขตร้อน)

บทคัดย่อ

การศึกษาครั้งนี้เป็นการพัฒนาวิธีตรวจโรคบิดอะมีบาโดยใช้วิธี Western blot ซึ่งตรวจหาแอนติบอดีที่จำเพาะต่อแอนติเจน EhPFOR ในเซรุ่มผู้ป่วยที่ติดเชื้อบิดอะมีบาโดยเทียบกับตัวตรวจสอบมาตรฐาน (positive control) ที่แบนด์น้ำหนักโมเลกุล 144 kDa ของโมโนโคลนอลแอนติบอดีของ *โคลน* Eh208C2-2 ซึ่งทำปฏิกิริยาจำเพาะกับแอนติเจน EhPFOR ผลการทดสอบในเซรุ่มทั้งหมด 70 ราย แบ่งเป็น 3 กลุ่ม กลุ่มที่ 1 มี 50 ราย แบ่งเป็นกลุ่มย่อย 1A 30 ราย เป็นผู้ป่วยที่มีอาการ ตรวจอุจจาระด้วยกล้องจุลทรรศน์พบเชื้อบิดอะมีบาและให้ผลบวกเมื่อตรวจทางเซรุ่มด้วยวิธี IHA, IEP สามารถตรวจพบแบนด์ 144 kDa ชัดเจนทุกราย กลุ่มย่อย 1B 20 รายเป็นผู้ป่วยที่ไม่แสดงอาการและตรวจอุจจาระพบเชื้อบิดอะมีบาก่อโรค/ไม่ก่อโรคด้วยกล้องจุลทรรศน์เพียงวิธีเดียว ตรวจพบแบนด์ 144 kDa เพียง 7 ราย ส่วนกลุ่มที่ 2 เป็นผู้ป่วย 10 รายที่ตรวจพบโปรโตซัวชนิดอื่นในอุจจาระได้แก่ *E. coli* (4ราย), *E. nana* (2 ราย), และ *B. hominis* (4ราย), และกลุ่มที่ 3 เป็นกลุ่มควบคุม 10 รายตรวจอุจจาระแล้วไม่พบเชื้อโปรโตซัวทุกชนิดและตรวจไม่พบแบนด์ 144 kDa นอกจากนั้นเซรุ่มของผู้ป่วยกลุ่ม 2 จำนวน 1 ใน 4 รายที่ติดเชื้อ *B. hominis* และ จำนวน 1 ใน 4 รายที่ติดเชื้อ *E. coli* ตรวจพบแบนด์อื่นที่น้ำหนักโมเลกุล 115, 83 และ 59 kDa ตามลำดับ หากใช้การตรวจเชื้อด้วยกล้องจุลทรรศน์หรือ การตรวจเซรุ่มเป็นวิธีมาตรฐานวิธี Western blot ให้ค่าความไวและค่าประสิทธิผลร้อยละ 100 ร้อยเท่ากันในกลุ่มย่อย1A และ ร้อยละ 35 และ 56.6ในกลุ่มย่อย1B โดยทั้ง 2 กลุ่มให้ค่าความจำเพาะร้อยละ100 เท่ากัน สรุปได้ว่าสามารถนำวิธี Western blotting มาประยุกต์ใช้ในการวินิจฉัยยืนยันการติดเชื้อบิดอะมีบาได้ดี โดยเฉพาะอย่างยิ่งเมื่อมีเชื้ออะมีบาระยะบุกรุกและแพร่กระจายไปที่ตับ

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CONTENTS

CONTENTS (continued)

LIST OF TABLES

Tables **Page**

LIST OF FIGURES

Figures **Page**

LIST OF ABBREVIATIONS

ABBREVIATIONS	TERM
ALA	Amoebic liver abscess
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
C	Complement
CAP	Cellulose Acetate Precipitin
$CoCl_2$	Cobalt chloride
DAB	Diaminobenzidine
DADE	Diethylaminoethyl
DMSO	Dimethyl sulfoxide
DW	Distill water
E. dispar	*Entamoeba dispar* (Ed)
E. histolytica	*Entamoeba histolytica* (Eh)
ELISA	Enzyme-linked immunosorbent assay
et al	Et alii: and other
FICT	Fluorescein isothiocyanate
GAM	Goat anti-mouse immunoglobulin
H_2O_2	Hydrogen peroxide
HM-1: IMSS	A strain of *E. histolytica*, Human Mexico No.1,Instituto Mexicano del Seguro Social
HRP	Horse radish peroxidase
i.e.	id est: that is
IFA	Indirect immuno fluorescent assay
Ig	Immunoglobulin
IHA	Indirect heamagglutination
kDa	Kilodalton (s)

LIST OF ABBREVIATIONS (continued)

ABBREVIATIONS	TERM
LPGs	Lipophosphoglycans
MAb	Monoclonal antibody
min	minute
MW	Molecular weight
NSS	Normal saline solution
PAGE	Polyacrylamide gel Electrophoresis
PAHO	Pan-American Health Organization
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline with 0.05% Tween 20
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PFOR	Pyruvate ferredoxin Oxidoreductase
RBC	Red blood cell
RBCs	Red blood cells
rpm	Revolution per minute
SDS	Sodium dodecyl sulfate
SOD	Super oxide dismutase
V	Volt (s)
WHO	World Health Organization
µl	Micro liter (s)
µm	Micrometer (s)

CHAPTER 1
INTRODUCTION

Amoebiasis refers to human infection of *E. histolytica* in reference to the species capable of causing invasive disease (formerly known as pathogenic *E. histolytica*) with or without clinical manifestation (WHO/PAHO/UNESCO, 1997). *E. histolytica* is one of the ten most common infections in the world today. In 1984, 500 millions people were infected with *E. histolytica* and about 40-50 millions developed clinical amoebiasis each year, resulting in up to 100,000 deaths (Walsh, 1988).

Member of all age groups and both genders are infected, but there is a higher prevalence of amoebiasis among adult men, who have an increase risk of exposure in the agricultural occupations (Despommier *et al*.,1995). An increased prevalence of this infection is also found in male homosexual (Pomerantz *et al.,* 1980; Haghighi *et al.,* 2002 and Haghighi *et al.,* 2003), although this protozoan may be less pathogenic in this population (Goldmeier *et al.,* 1986). Animals other than human such as dogs, pigs, cats, rats and monkeys can be incidental hosts, dogs and cats can be naturally infected hosts. However, the studies for the real prevalence's of *E. histolytica* and *Entamoeba dispar* are scanty. This discrepancy between high prevalence and low morbidity is explained by two species theory; pathogenic *E. histolytica* and non-pathogenic *E. dispar*. The two species are morphologically identical, but are different by immunologic, biochemical, pathogenic and genetic characteristics (Clark and Diamond, 1993). Since infection by *E. dispar* is known to be non-harmful, it is practically necessary to distinguish it from that of *E. histolytica* (Tannich and Buchard, 1991)

Entamoeba has been described as a facultative aerobe in that it can grow in 5% oxygen which oxygen breakdown products are toxic to *Entamoeba*. The parasite produces super oxide dismutase (SOD), catalase and peroxidase for detoxification.

E. histolytica responsible for the human amoebiasis, the oxidative decarboxylation of pyruvate is catalyzed by the pyruvate ferredoxin oxidoreductase (PFOR) enzyme (Kerscher and Oesterhelt, 1982).

Metronidazole [1-(2-hydroxyethyl)-2methyl-5 nitroimidazole)] is the drug of choice for the treatment of infections cause by anaerobic or microaerophilic microorganism (Freeman *et al.*, 1997). The drug enters the cell through passive diffusion, where a nitro group is subsequently reduced to reactive cytotoxic nitro radicals by reduced ferredoxin or flavodoxin (Wassmann *et al.,* 1999). Ferredoxin and flavodoxin function as electron acceptors of PFOR resulting in the cytotoxic activation of metronidazole. Decreased pyruvate: ferredoxin oxidoreductase (PFOR) activity in anaerobic organisms is one mechanism of metronidazole resistance but in *Entamoeba*, PFOR activity was not decreased in metronidazole-resistant parasites as determined by immunofluorescent assays and immunoblotting studies. A marked increase in superoxide dismutase (SOD) activity was detected in metronidazole-resistant *E. histolytica* (Samarawickrema *et al.,* 1997). In metronidazole-resistant lines of *Trichomonas vaginalis* (Johnson, 1993), *Bacteroides fragilis* and *Clostridium perfringens* (Sindar *et al.,* 1982) decreased PFOR activity is associated with resistance (Townson *et al*., 1994). However, in case of *Tritrichomonas foetus* a complete absence of PFOR is correlated with resistance to high levels of metronidazole (Johnson, 1993).

Many of the glycolytic enzymes are responsible and utilize the energy of the pyrophosphate bond, not ATP. Only one 2-oxoacid oxidoreductase, PFOR has been detected in *E. histolytica.* PFOR is a key enzyme not only in the energy production pathway of *E. histolytica*, but also by its role in the reduction of the nitrogen group of metronidazole in **Figure 1**. Data from Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of anti-*E. histolytica* monoclonal antibodies (Eh208C2-2 MAb) under non-reducing condition indicated that anti-EhPFOR MAb is duplex of molecular weight 125 kDa and 140 kDa (Sosa, 1994). Data from Immunoblot studied by Samarawickrema *et al.,* (1997) indicated that *E. histolytica* PFOR is approximately 140 kDa. Which is consistent with the size of the EhPFOR gene (Genbank accession

number L46793, Thammapalerd *et al.,* 1996a) and that predicted by Rodriguez *et al*., (1998) 130 kDa. Since EhPFOR is an abundant, highly conserved an immunogenic protein (Thammapalerd *et al.,* 1996a), therefore, the presence of antibody to PFOR should be evidenced in the sera of amoebiasis patients.

SIGNIFICACE OF THIS STUDY

Intestinal amoebiasis is caused by human infection with *E. histolytica*. The symptoms vary from asymptomatic to amoebic dysentery. Extraintestinal amoebiasis involves the invasion of intestinal mucosa by the parasites beyond the mucosa to other organs including liver and brain. The examination of fresh stool for the presence of the cysts and/or trophozoites is mandatory and should be carried out as soon as the diagnosis is suspected. Microscopic examination of stool for amoebae is difficult procedure requiring considerable technical expertise. False positive results, in which polymorphonuclear leukocytes, macrophages, or *Entamoeba coli* are mistaken for *E. histolytica*, as common as false negative results, in which amoebae fail to be recognized. Antibody detection, serologic testing for intestinal disease is normally not recommended unless the patient has true dysentery; even in the titer (indirect hemagglutination as an example) may be low and thus difficult to be interpreted (Filice *et al*., 1993).

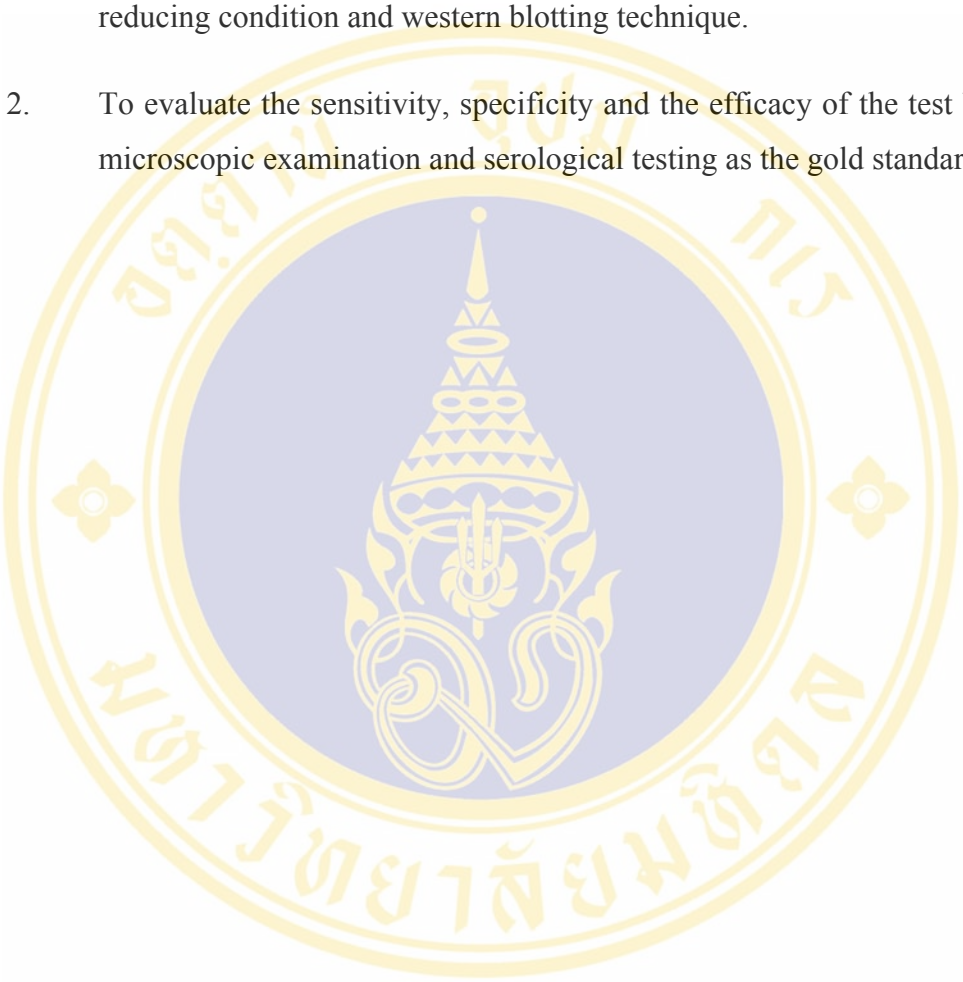
Mouse monoclonal antibodies (MAbs) have been produced successfully against 3 pathogenic strains of *E. histolytica* at the Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University. One MAb, in particular Eh208C2-2, was reactive against EhPFOR (Thammapalerd *et al.,* 1996a) and has been applied to detect amoebic antigen in clinical specimens of humans and hamsters (Wonsit *et al.,* 1992; Thammapalerd *et al.,* 1996b; Sherchand *et al.,* 1994; Thammapalerd *et al.,* 1996c). The present study was designed, therefore, to identify anti-PFOR antibody against EhPFOR specific parasite antigens and immunogenic to the human host suffering from intestinal and extraintestinal amoebiasis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) allows separation of antigenic fraction of *E. histolytica* followed by the Western blotting procedure with

sera allowing the identification of specific and immunogenic fraction of anti-PFOR antibody which should be appeared only in patients' sera and that of Eh208C2-2 MAb when compared with those of patients' infected with parasites other than *E. histolytica* and healthy control sera. This analysis technique can be applied for immunodiagnosis of invasive amoebiasis among the other assays.

SPECIFIC OBJECTIVE

1. To develop an immunoassay for the detection of anti-amoebic PFOR antibody in amoebiasis patients' sera by using SDS-PAGE under non-reducing condition and western blotting technique.

2. To evaluate the sensitivity, specificity and the efficacy of the test by using microscopic examination and serological testing as the gold standard.



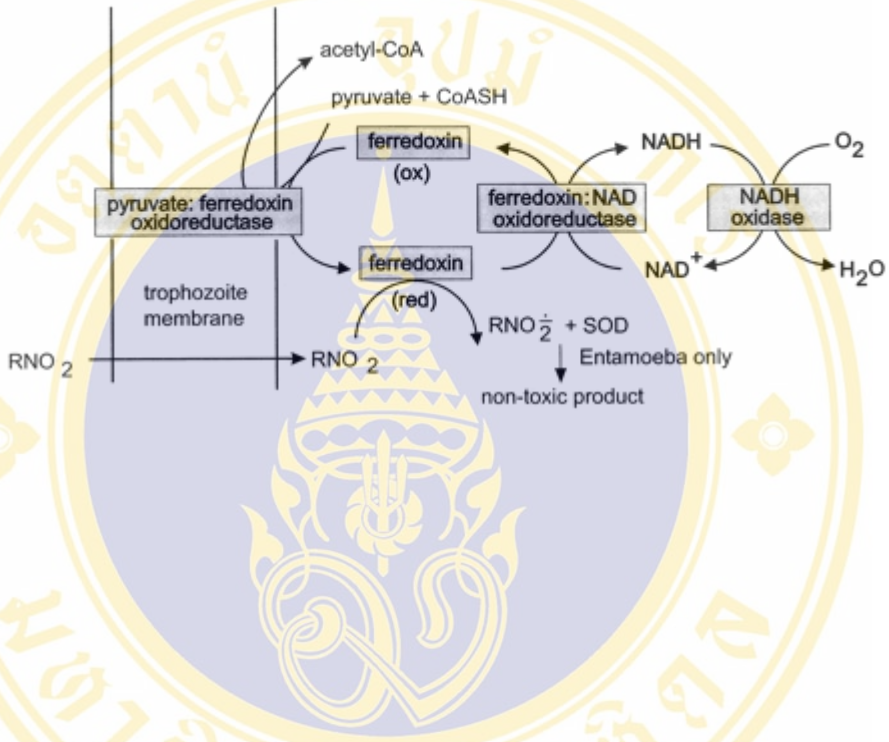


Figure 1. Proposed fundamental metabolism of the anaerobic protozoa, with PFOR replacing the pyruvate dehydrogenase of aerobic organisms (Upcroft and Upcroft, 2001).

CHAPTER 2
LITERATURE REVIEW

2.1 Amoebiasis

Amoebiasis refers to human infection of *E. histolytica* in reference to the species capable of causing invasive disease (formerly known as pathogenic *E. histolytica*) with or without clinical manifestation. It is responsible for approximately 100,000 deaths per annum worldwide, second in importance only after malaria among the pathogenic protozoa (WHO/PAHO/UNESCO, 1997).

2.2 Toxonomy of *E. histolytica*

The superclass Sarcodina evolved from Mastigophora, but, as traditionally conceived, the group is polyphyletic (Levine *et al.,* 1980). Of the many families of amoebas, only the Endamoebidae has a species of great medical or economic importance. The species in the Endamoebidae are parasites or commensally of the digestive systems of arthropods and vertebrates on the basis of nuclear structure. Three genera contain known parasites or commensally of humans and domestic animals: *Entamoeba, Endolimax*, and *Iodamoeba*. Species of *Entamoeba* are found in both vertebrate and invertebrate host. Five species are common in human beings (*E. histolytica*, *E. dispar*, *Entamoeba hartmani, E. coli, and Entamoeba gingivalis*).

2.3 Morphology and Life cycle

The morphology and life cycle of *E. histolytica* are shown in **Figure 2 and Figure 3** respectively. The mature 4 nucleated cyst forms are infective stage for humans.

Trophozoites

Living trophozoite vary in the size from about 10 μm to 60 μm in diameter. Organisms recovered from diarrheic or dysenteric stools are generally larger than those in a formed stool from asymptomatic individual. The motility has been described as rapid and unidirectional, with pseudopods forming quickly in response to the condition around the organism. The motility may appear to be sporadic. Although this characteristic motility is often described, it is rare to diagnose amoebiasis on the basis of motility seen in a direct mount. The cytoplasm differentiates into a clear outer ectoplasm and a more granular inner endoplasm.

When the organism is examined on a permanent stained smear (trichrome or iron hematoxylin), the morphological characteristic are readily seen. In organisms isolated from a patient with dysentery, red blood cell may be visible in the cytoplasm, and this feature is usually diagnosis for *E. histolytica*. Most often, infection with *E. histolytica* will be diagnosed on the basis of organism morphology without the presence of red blood cells (Garcia and Bruckner, 1997).

Cysts

The trophozoites may condense into a round mass (precyst), and a thin wall is secreted around the immature cyst. There may be two types of inclusion within this immature cyst, a glycogen mass and highly retractile chromatoid bars with smooth, rounded edges. As the cysts mature (metacyst), there is nuclear division with the production of four nuclei, range in size commonly 10 μm to 20 μm. Often, as the cyst matures, the glycogen completely disappears.

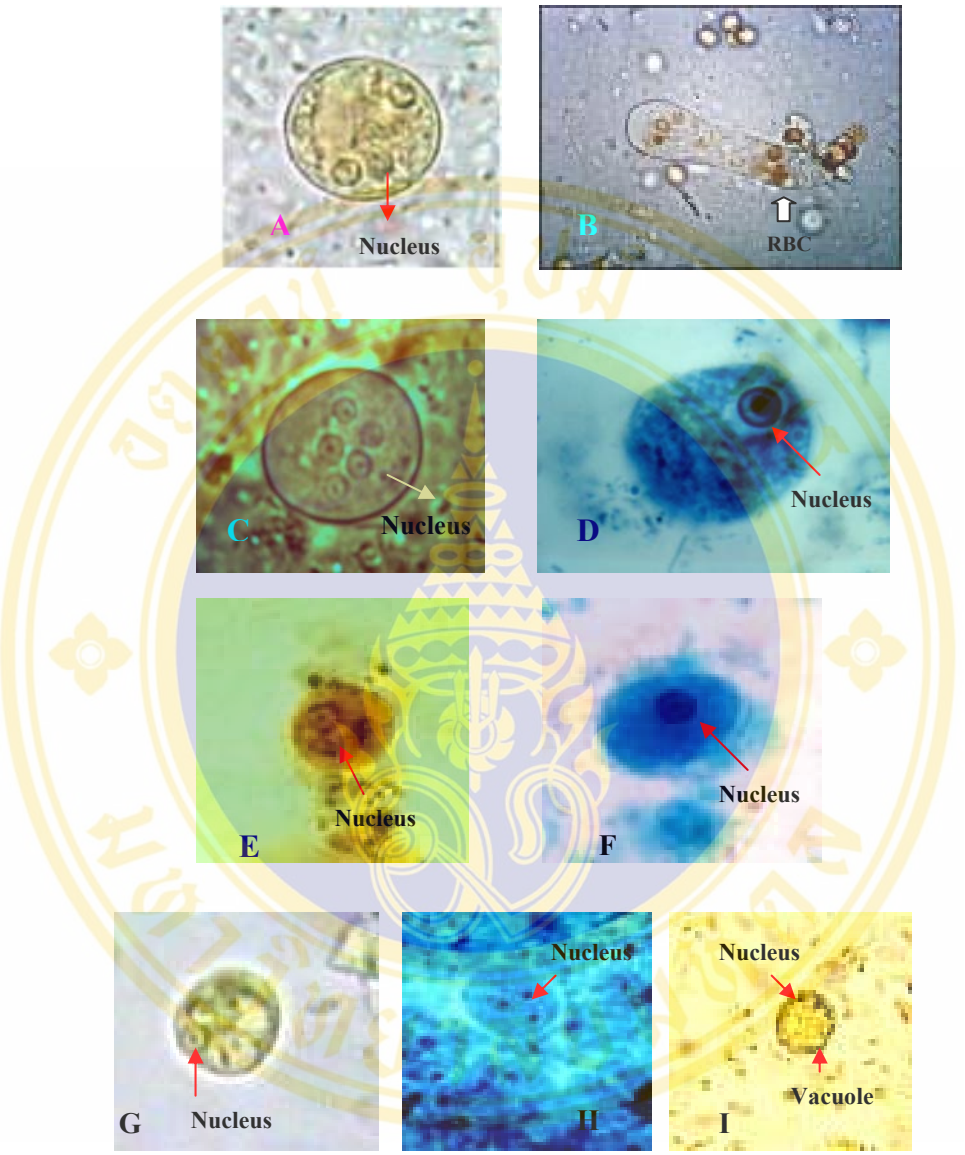


Figure 2. Morphology of protozoal parasites; *E. histolytica* mature cyst with 4 nuclei and trophozoite ingested red blood cells in NSS (A and B), *E. coli* cyst stage with 1-8 nuclei stained with iodine (C) and trophozoite stage with stained trichrome (D), *E. hartmanni* cyst stage and trophozoite stage stained with trichrome (E and F), *E. nana* cyst in NSS from fresh stool specimen (G),Trophozoite stage *E nana* in stool stain with Iron-Hematoxylin (H), *B. hominis* vacuolated form by stained with iodine (Radomyos *et al.,* 1998).

Figure 3. Life cycle of *Entamoeba histolytica* and the clinical manifestations of infection in humans**.** *E. histolytica* is a protozoan parasite that causes amoebic colitis and liver abscess (Christopher *et al.,* 1999).

Infection

After cyst ingestion by humans, no changes occur in an acidic environment of the stomach; however, once the pH become neutral or slightly alkaline, the encysted organism becomes active with the outcome being four separated trophozoites (small, metacystic trophozoites). These organisms develop into the normal trophozoites when they become established in the large intestine (Bruckner, 1992).

2.4 Pathology and Pathogenesis

Pathogenic and non-pathogenic *E. histolytica* actually comprise two morphologically identical species, differing genetically and in their capacity to cause disease. One species, *E. histolytica* is an invasive pathogen exhibiting easing degree of virulence, the other as a non-invasive *E. dispar* which at the most may be capable of producing superficial erosions of the colonic mucosa and is recognized by Brumpt since 1925 (Warhurst, 1999).

A number of virulence factor derived from *E. histolytica* that mediate the pathogenic effect of amoebiasis (Ravdin, 1989), such as an adherence lectin, a pore-forming peptide lysing host cells and protease degrading host tissue (Ackers, 1996). Pathogenesis starts from the attachment of the amoebic trophozoite to host cell by the parasite which is mediated by a lectin molecule that binds to galactose-and N-acetyl-D-galactosamine (Petri *et al*., 1987). It is the most prominent antigen recognized by immune sera from amoebiasis patients (Petri *et al.,* 1987). In addition, *E. histolytica* release several factors that promote tissue invasion and cytotoxicity. One is a cysteinyl protease, histolysain, that degrades the extra cellular matrix (Keene *et al.,* 1986) and this enzyme can also activate complement (Reed *et al.,* 1989). A second factors is a pore-forming lipophilic protein that lyses target cell by creation holes in membrane. A third factors is a phospholipase that promotes cytolytic activity (Young *et al*., 1982). A family of amoebic glycoconjugates sharing some structural similarities with the lipophosphoglycan (LPGs) of *Lieshmania* (McConville, 1991;

Turco and Descoteaux, 1992) has also been recently identified in *E. histolytica* and proposed to contribute to amebic adhesion and cytotoxicity.

2.5 Clinical Diseases

The presentations of disease are seen with invasion of the intestinal mucosa and /or dissemination to other organs, which the most common is the liver. However, it is estimated that only a small proportion (2 to 8 %) of infected individuals will have invasive disease beyond the lumen of bowel. Also, organisms may be spontaneously eliminated with no disease symptoms (Garcia and Bruckner, 1997).

Asymptomatic Infection

Individuals harboring *E. histolytica*, may have titer a negative or weak antibody titer and negative stools for occult blood, may be passing cysts that can be detected if routine ova and parasite examination is performed. Although trophozoites may be found, they will not contain any phagocytized RBCs. Isoenzyme analyses of organisms isolated from asymptomatic individuals generally indicate that the isolates belong to nonpathogenic zymodemes (Wilson *et al.,* 1995) which is now equivalent to *E. dispar*. Asymptomatic cyst passage is the most common manifestation of intestinal *E. histolytica* infection. Generally, asymptomatic patients never become symptomatic and may excrete cysts for short period. This pattern is found for patients infected with either non-pathogenic or pathogenic strains (Diamond and Clark, 1993).

Intestinal Diseases

Microscopy studies suggest that amoebae have enzymes which lyses host tissue, possibly from lysosome on the surface of the amoebae or from ruptured organism. From these primary sites, other lesions may occur. Ulcers are usually rise with a small opening on the mucosal surface and a larger of destruction below the surface, i.e. “flask shaped” (Brandt and Tamayo, 1970). .

Patients with amoebic colitis usually note the gradual onset of abdominal pain and watery stool contain mucus and blood. 80% of patients complain localized abdominal pain (Adam *et al.,* 1997). If left untreated, a number of patients spontaneously resolve their symptoms. Eroded ulcers surrounded by normal appearing epithelium characterize amoebic colitis. Fulminate colitis with sever bloody diarrhoea, fever, and abdominal pain, is usually a pediatric disease. An unusual complication of intestinal infection, 60 % of patients progresses to colonic perforation. The most serious complication of amoebic colitis is perforation and secondary bacterial peritonitis, which may present acutely or subacutely (Adam *et al.,* 1997).

Extraintestinal Amoebiasis

Amoebic liver abscess is the most common manifestation of extraintestinal disease because blood flow draining the intestine tends to return to the liver, most commonly the upper right lobe. The organisms present in the submucosa can therefore be carried via the blood stream to the liver (Garcia and Bruckner, 1997). The clinical presentation is highly variable, ranging from obvious infection of the liver, to prominent pulmonary symptoms.

Trophozoites reach the liver through the portal venous system causing periportal inflammation. To survive dissemination through the blood stream trophozites are resistant to the lytic action of complement. Amoebic liver abscess (ALA) tend to be hepatomegaly with tenderness; however, liver function tests may be normal or slightly abnormal, with jaundice being very rare. There may be changes at the base of the right lung owing to the elevated diaphragm (Allison *et al.,* 1986). The most common complication is rupture of the abscess into the pleural space. An abscess can also extend into peritoneum and through the skin, hematogenous spread to the brain, as well as to the lung, pericardium, and other site, is possible. As with other invasive protozoal diseases, eosinophilia is not a feature. The prevalence, risk factors and maternal-perinatal in 91 cases consequences of chronic asymptomatic intestinal parasitic infection with *E. histoly*tica were present 88% of maternal iron deficiency anemia and fetal growth retardation.

The only consistent abnormality in blood chemistries is in elevated alkaline phosphatase, which is present in more than 75% of patients with amoebic liver abscesses. Elevated transaminases are present only in patients who present acutely or with multiple abscesses (Warhurst, 1999).

2.6 Diagnosis of Amoebiasis

Microscopic Diagnosis

The identification of hematophagous trophozoites is the hallmark of *E. histolytica* infection. Examination of the fresh stool or scrapings from an ulcer for motile trophozoites is optimal. Examination of trichrome-stained concentrates of three fresh stools is also important. In amoebic liver abscesses, trophozoite are usually only present in the capsule and are rarely detection by slides, but is not routinely available. *E. histolytica* and *E. dispar* are morphologicall identical and cannot be differentiated by microscopy alone without the presence of phagocytosed erythrocyts in *E. histolytica*. A recent WHO Special Committee recommended that quadrinucleated cysts now be identified and reported as *E. histolytica*/*E. dispar* (WHO, 1997).

Histology

A histological diagnosis of amoebiasis can be made when the trophozoites within the tissue are identified. Organisms must be differentiated from host cells, particularly histiocytes and ganglion cells. Periodic acid-Schiff staining is often used to help locate the organisms. The organisms will appear bright pink with green-blue background. Hematoxylin and eosin staining will also allow the typical morphology to be seen, thus allowing accurate identification (Garcia and Bruckner, 1997).

Immunodetection

Although reagent development has been under way for some time, earlier attempts at immunodetection of organisms in stool samples were marginally successful. The level of sensitivity and specificity of these reagents was no greater than that of routine stool examination. However, more recent work indicates definite improvement in reagent quality (Broaddus *et al.,* 1985).

To provide clinically relevant information to physicians for treatment of patients infected with pathogenic strain of *E. histolytica*, methods involving monoclonal antibodies, purified antigens, or DNA probes would be helpful. Reagents have been developed to differentiate pathogenic *E. histolytica*, from non pathogenic *E. dispar* (Reed *et al.,* 1991). Although some of these reagents are now available, routinely used in clinical laboratories may not be accepted because of cost, limited number of cases, and infrequent identification of organism. Reliable probes based on the hypotheses of zymodeme stability and two separate species (one pathogenic, one nonpathogenic) offer significant improvements over current serologic or direct detection method; these probes may also help eliminate false-positive results due to misidentification of human cell or other nonpathogenic protozoa in patient specimens.

An ELISA test is now available for the detection of the adherence protein of *E. histolytica* and will enhance our capability to detect invasive disease. Using antibody against galactose-inhibitable lectin, Abd-Alla and colleagues were able to detect *E. histolytica* adherence protein antigen directly in serum and fecal sample by ELISA. The presence of amoebic antigen in serum demonstrated 94% specificity for pathogenic *E. histolytica* infection, and amoebic antigen was present during symptomatic intestinal infection (Abd-Alla *et al.,* 1993).

Invasive-specific monoclonal antibodies may be used in indirect immunofluorescence tests to confirm the presence of *E. histolytica* trophozoites in culture (Gonzalez-Ruiz *et al.,* 1992). Polymerase chain-reaction solution

hybridization enzyme-linked immunoassay (PCR-SHELA) was used for the differential diagnosis of pathogenic *E. histolytica* and non-pathogenic *E. dispar* (Aguirre *et al.,* 1995) and appeared to be a user-friendly approach with high specificity and sensitivity.

Antibody Detection

Serological tests (Maddison, 1991) are valuable to detect extra-intestinal disease, where antibody levels are raised on presentation in 95% or more of cases. In endemic areas, the distinction between background levels of antibody in currently uninfected persons and raised levels in persons suffering from intestinal disease is often not clear in up to 50% of cases (Shetty *et al.,* 1988).

An ELISA test for anti-amoebic antibody was evaluated for diagnosis of amoebic liver abscess, the test parameters were: sensitivity, 95.7%; specificity, 100%. The ELISA test was found to be valuable as a diagnostic tool for the establishment of amoebic etiology in suspected ALA patients (Nicholls *et al.,* 1994). An ELISA test using recombinant serine-rich fusion protein (expressed with maltose-binding protein) had sensitivity of only 74% and specificity of only 55% in liver abscess.

Precipitin tests, in particular the cellulose acetate precipitin test (CAP) (Stamm and Phillips, 1997; Thammapalerd *et al*., 1981), have proved useful; the CAP in particular generally becomes negative 3 months after treatment. Although studies of amoeba-specific IgM have not generally shown it a useful indicator of active disease (Osisanya and Warhurst, 1980), a more recent report on hepatic amoebiasis, which includes patients diagnosed with non-suppurative hepatic amoebiasis, a pre-abscess condition, suggests this may be a valuable diagnostic indicator (Baveja *et al.,* 1992).

As dot immunobinding assays are easier to perform than ELISA, equally sensitive and specific and more economical on antigen, they have been recommended for the detection of antibodies in patients with suspected liver abscess or other forms of extra-intestinal amoebiasis (Baveja *et al.,* 1991). The technique has been used for

detection of salivary IgA antibody (Punthuprapasa *et al*., 2001) sIgA in colostrums (Lopez-Revilla *et al.,* 1991). The serology for antibody may or may not be positive in intestinal disease and is much more likely to be positive in extra intestinal disease (Warhurst, 1991). Indirect hemagglutination (IHA) and indirect fluorescence antibody (IFA) tests have been reported with titers of 1:256 and 1:200, respectively, in almost 100% of case in amoebic liver abscess (Katzenstein *et al.,* 1982).

2.7 Treatment

Metronidazole is the drug of choice for patients with acute amoebic dysentery or for extraintestinal invasive; it can be given in equivalent dose orally or intravenously (Reed, 1992). Even with appropriate therapy, however, liver abscesses usually resolve slowly. Metronidazole also has a number of side effects. Because of its potential hepatogenicity, the use of Metronidazole during pregnancy cannot be recommended unequivocally. Metronidazole has questionable efficacy against the cyst stages of *E. histolytica*. Iodoquinol is a relatively nontoxic drug, but it occasionally causes rash, an exacerbation of exfoliate dermatitis (McAuley and Juranek, 1992).

Certain form of intestinal and liver amoebic lesions may require surgical treatment where there is serious involvement (Guarner, 1986). The role of open surgical drainage or percutaneous drainage using ultrasound guidance (Citronberg and Semel, 1995). Patients receiving combined guided drainage and intralesional chemotherapy experienced a faster and overall better clinical response, which was confirmed by sonographic follow up of the hepatic lesions (Filice *et al.,* 1992).

2.8 Epidemiology, Prevention and Control

Infections with *E. histolytica*, are worldwide in distribution and are generally most prevalent in the tropics. Population groups that have been found a higher incidence of amoebiasis (Guerrant, 1986) include recent immigrants and refugees from South and Central America and from Southeast Asia. Residents in southeastern and southwestern parts of the United States also tend to have more infections with intestinal parasites. In Thailand, the point prevalence varied from 1.4-4.2% (Bunnag *et al*., 1971).

Following broad social changes seen in the late 1960s, the open expression of homosexuality, increased numbers of sexual contacts, increased frequency of sexual activities, and anonymity of sexual partners contributed to dramatic increased in sexually transmitted organisms, including *E. histolytica*. Although infection with this organism are usually associated with poor sanitation and underdeveloped areas of the world.

There are certain urban areas within the world where the incidence of invasive disease is considerably higher than in the rest of the world. Contributing factors may include poor nutrition, tropical climate and decreased immunologic competence of the host. The human is the reservoir host for *E. histolytica* and can transmit the infection to other humans. The cyst stages are very resistant to environmental conditions and can remain viable in the soil. The asymptomatic cyst passer who is a food handler is generally thought to play the most important role in transmission (Garcia and Bruckner, 1997).

Health education action made in 1991 by the informal WHO/PAHO Committee (WHO, 1991) should be integrated into the community for a better prevention and control. It has been noted that in endemic area the recurrence of hepatic abscess after successful treatment is rare, and the same applies to severe amoebic colitis. The report of intestinal colonization without symptoms in hepatic

abscess, mentioned elsewhere, supports an immune response in humans, developing after symptomatic recovery, which protects against invasion in the gut.

2.9 Immunity, Immusupression and Immunopathology

Evidence for some immunosupression by *E. histolytica* has been obtained, and it has been shown that responsiveness to mitogen in murine lymphocytes is down-regulated in mice injected with *E. histolytica* antigens as opposed to those injection with 'Eh'(Laredo) antigens. Secreted cysteine protease appears to be involved in complement alternative pathway activation in *E. dispar* and nonvirulent *E. histolytica* by leaking down C3 and C5 to anaphylotoxins C3a and C5a (Reed *et al.,*1995). However, extracellular cysteine proteinase of virulent *E. histolytica* apparently further degrades and inactivates these proinflammatory factors, circumventing normal host immunity (Reed *et al*.,1995). Although amoebic proteins by themselves are incapable of stimulating the respiratory burst response of macrophages, they are capable of modulating it, suggesting an important role in the immunoregulation and pathogenesis of amoebiasis (Lin *et al.,* 1993). An increase in amoeba binding to enterocytes is provoked by cytokine treatment *in vitro* which suggests that the parasite may take advantage of inflammatory stimuli in order to increase its binding to colonic epithelium (Flores-Romo *et al.,*1993).

2.10 Monoclonal Antibodies

A monoclonal antibody is defined as a uniform homogeneous antibody directed at a single epitope or antigenic determinant and produced continuously from one cell clone. In 1975, Köhler and Milstein first reported a successful method of producing monoclonal antibodies. They fused a specific antibody-producing cell with a mutant myeloma cell line to produce a hybridoma, which resulted in immortalization of the specific antibody-producing cell. The technology introduced by Köhler and Milstein has led to an explosive application of monoclonal antibodies in research and clinical diagnostic medicine. The use of monoclonal antibodies in clinical laboratories has been established in numerous assays.

The most useful out growth of monoclonal antibody technology has been the development of many new highly specific and sensitive immunoassay formats. This technology will allow the development of an ideal immunoassay system for the clinical laboratory. (Carayanniotis and Barber, 1987).

Some of common applications of monoclonal antibodies and hybridomas include the following: (Abbas *et al.,* 1994)

1. Identification of phenotype markers unique to particular cell types. The basis for the modern classification of lymphocytes and other leukocytes is the binding of population-specific monoclonal antibodies. These have been used to define "clusters of diffentiation" (CD markers) for various cell types.
2. Immunodiagnosis. The diagnosis of many infectious and systemic diseases relies on the detection of particular antigen and/or antibodies in the circulation or in tissues, using monoclonal antibodies in immunoassays.
3. Tumor diagnosis and therapy. Tumor-specific monoclonal antibodies are used for detection of tumors by binding imaging techniques and for immunotherapy of tumor *in vivo*.
4. Functional analysis of cell surface and secreted molecules.

The most useful out growth of monoclonal antibody technology has been the development of many new highly specific and sensitive immunoassay formats. This technology will allow the development of an ideal immunoassay system for the clinical laboratory. (Carayanniotis and Barber, 1987).

Conventional characterization of anti-*E. histolytica* MAb will be restricted to three methods comprising IFA-staining patterns (1) showing positivity against to pathogenic *E. histolytica* combined *E. histolytica*-like (Laredo) and non-*E. histolytica* amoebae (negative reactivity). Based on IFA staining patterns could be divided arbitrarily into 5 groups **(Appendix D)**. Agglutination (2); a simpler agglutination technique by mixing a MAb with trophozoites were shown to agglutinate live amoebic trophozoites and (3) inhibition of adherence of amoebic trophozoites to target cells (Thammapalerd *et al.,* 1991).

2.11 SDS-PAGE and Western blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is the common used laboratory technique for separating proteins detection with stains. The method can also be used for the small-scale purification of protein. SDS in the presence of a reducing agent (2-mercaptoethanol) is a technique for the separation of polypeptide subunits according to their molecular weight (Shapiro, 1967). The protocol involves denaturing the protein sample by heating in the presence of SDS and a reducing agent. SDS will bind to the protein causing it to unfold, whereas the reducing agent will reduce the intramolecular and intermolecular disulfide bonds. The binding of SDS by protein confers a net negative charge and the denatured polypeptide will migrate through a gel known percent acrylamide in the presence of an applied electric field towards the positive electrode (anode). Therefore, in reducing SDS-PAGE, protein subunits of known molecular mass can be identified predictably and reference to a set of molecular weight standards that are electrophoresis. If reducing agent is not added to the protein sample containing SDS, covalent intra-polypeptide bonds in proteins remain intact. The result protein those are neither linearized nor necessarily reduced to individual polypeptides. Consequently, protein sample migrate

according to size in a manner that may have little correspondence to relative mass or molecular weight. After the electrophoresis is complete, the gel is stained with Coomassie Blue R-250 to visualize the polypeptide bands. A linear relationship exists between the log of the molecular weight of a polypeptide and it's R_f. By measuring R_f, that is, the ratio of the distance from the top of the gel to the polypeptide divided by the distance from the top of gel to the dye front, a standard curve can be generated. The curve will show the R_f of standard polypeptide and the log of their molecular weighs. The R_f of the polypeptide to be characterized is determined in the same way, and the log of its molecular weight read directly from the standard curve (Weber and Osborn, 1969).

Western blotting used to determine the relative quantity and the molecular weight of protein within a mixture of protein or other molecules to a suitable membrane. The method most commonly used for the electrotransfer of proteins to nitrocellulose is that reported by Towbin *et al*., (1979).

In order to take advantage of this technique for the purpose of amino acid analysis or N-terminal sequencing, the proteins must be transferred to a membrane that is stable to the chemicals used in these analytical procedures. The mixture is the first subjected to analytical separation, typically by SDS-PAGE, so that the final positions of different proteins in the gel are a function of their molecular size. The array of separated proteins is then transferred from the separating polyacrylamide gel to a support membrane by capillary action (blotting) or by electrophoresis. The membrane acquires a replica of the array of separated macromolecule presence in the gel. The position of the protein antigen on the membrane can then be detected by binding of labeled antibody specific for that protein, thus providing information about antigen size and quantity.

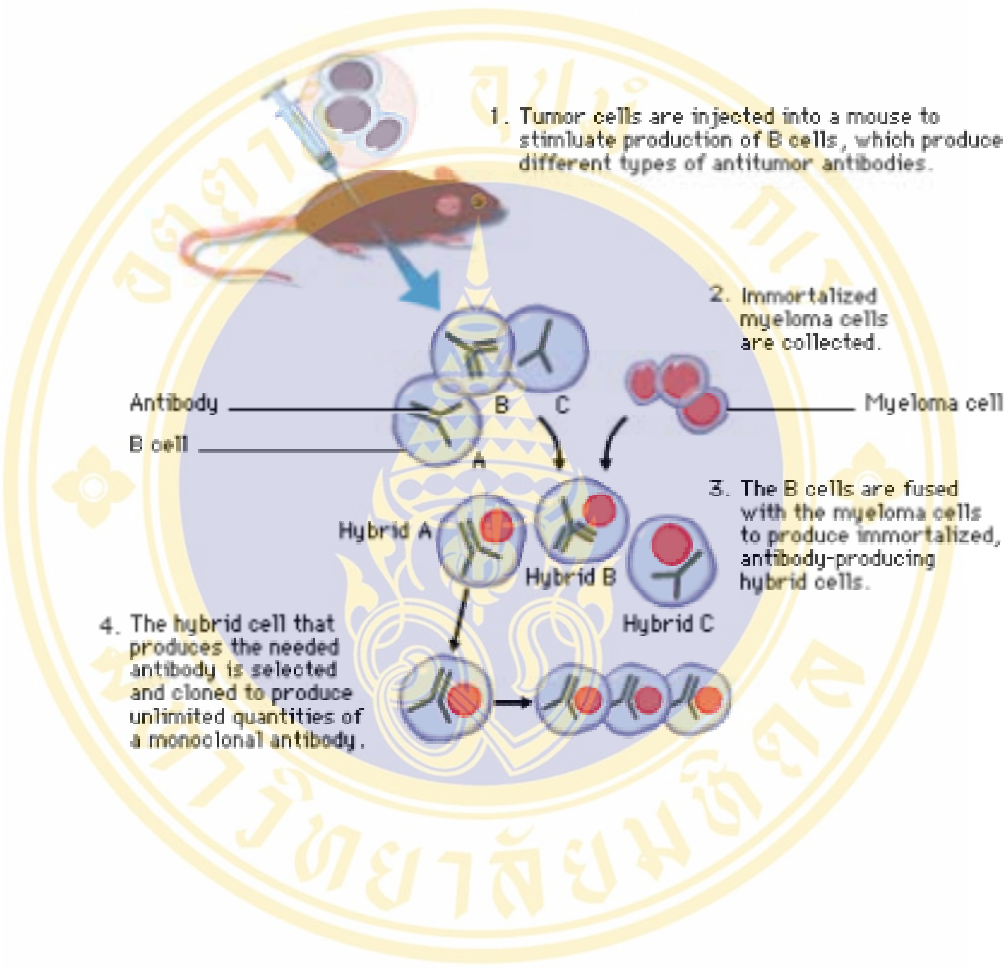


Figure 4. Monoclonal antibody production (Köhler and Milstein, 1975).

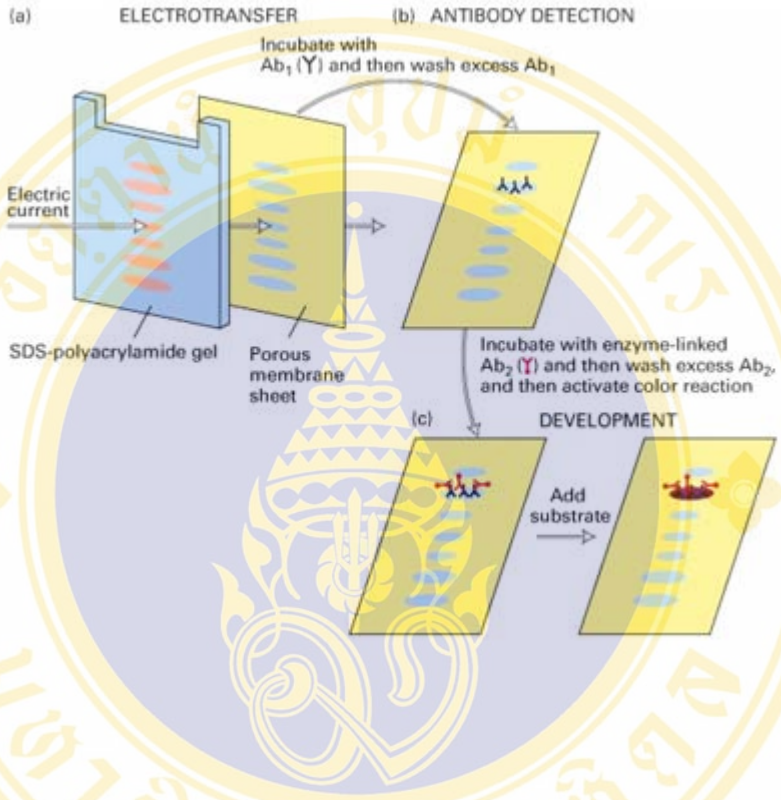


Figure 5. Process of Western blotting assay.

CHAPTER 3
MATERIALS AND METHODS

3.1 Preparation of *Entamoeba histolytica*

3.1.1 Parasite culture

The axenic *E. histolytica*, HM-1: IMSS was cultured using the technique described by Thammapalerd *et al.,* (1993). The cultured medium TYI-S-33 (Diamond *et al.,* 1978) **(Appendix A)** consisted of a nutrient broth (TYI), a vitamin-Tween 80 mixture and 10% heat inactivated sterile bovine serum. Subculture was done at 48-72 hour intervals. The old culture in the screw-capped test tube was chilled in ice bath for a 5-10 min to dislodge the amoebae adhering to the culture tube. The tube was inverted three times then centrifuged at 1500 rpm for 5 min. After centrifugation, the supernatant was discarded and the remaining sediment was transferred to a fresh medium.

3.1.2 Preparation of soluble *E. histolytica* antigen

The HM-1: IMSS strain of *E. histolytica* trophozoites axenically cultured from 48-72 hours were chilled to dislodge the parasites, followed by washing 3 times with 0.15 M normal saline solution (NSS) by centrifugation at 12,000 rpm at room temperature for 5 min. The cell sediment was adjusted in NSS to give approximately 10^7 cells/ml and disrupted at 4^{o}C with an ultrasonic disintegrator (MSE, London) at 20 kilocycles/min for 2 min. Complete disruption of cells was ascertained by examination under the light microscope. The cell lysate was centrifuged at 13,000 rpm for 20 min at 4^{o}C to remove associated cell residue and membrane particles. The opalescent supernatant was collected, dispensed in aliquots, lyophilized, metal capped, and stored (Wonsit *et al*., 1992). The protein content was determined by the method of Bradford protein

assay (Bradford, 1976) using bovine serum albumin (BSA) as a standard **(Appendix B)**.

3.2 Antisera

Seventy serum samples were obtained from 3 groups of patients. Group I was subdivided into Group IA and Group IB. Group IA composed of 30 sero- positive sera by serological tests from amoebiasis patients, Bangkok Hospital for Tropical Disease, Faculty of Tropical Medicine, Mahidol University and Institute of Pathology, Pramongkutklao Hospital, whose stools were positive for *E. histolytica* as determined by microscopic examination whereas Group IB composed of 20 patients' sera from different hospitals in Thailand whose stools were positive for *E. histolytica* as determined only by microscopic examination. Group II composed of 10 patients' sera whose stools were positive for other intestinal parasites such as *Entamoeba coli*, *Endolimax nana* or *Blastocystis hominis* and Group III composed of 10 healthy control sera from Institute of Pathology, Pramongkutklao Hospital whose stools were free from any intestinal parasites. The categories of patients' sera in this study are shown in **Table 1.**

3.3 Preparation of anti-*E. histolytica* Antibody

3.3.1 The Eh208C2-2 MAb

Mouse monoclonal antibody Eh208C2-2 (Eh208C2-2 MAb) specific to EhPFOR antigen was established at the Department of Microbiology and Immunology, Faculty of Tropical Medicine by Thammapalerd *et al.,* in 1986. The hybridoma secreting anti-PFOR MAb was prepared by fusion of Sp2/0 myeloma cells to the spleen cell of Balb/C mouse which was immunized with HM-1: IMSS strain of *E. histolytica*. The supernatant of Eh208C2-2 MAb was collected, concentrated by ammonium sulphate precipitation, IgG fraction separated and lyophilized.

3.3.2 Screening Assay for antibody against *E. histolytica* antigens

Dot-blotting for the screening method

Axenic trophozoites of *E. histolytica* strain HM-1: IMSS was used as antigens for re-testing the immunospecificity of Eh208C2-2 MAb. Antigens (1µl/spot) diluted in phosphate buffer saline (PBS) pH 7.5 and 2% sodium dodecyl sulfate-polyacrylamide gel (SDS) were applied to pieces of nitrocellulose membrane. The membrane was then baked at 60^0 C for 10 min, and blocked with 5% Blotto (Appendix G). Each piece was then incubated with Eh208C2-2 MAb and NSS used as negative control (1: 10 in 1% Blotto) for 2 hr and wash in 1% Blotto for 4 times with 15 min interval. The membrane was incubated in goat anti-mouse immunoglobulin-horseradish peroxidase conjugate (GAM-HRP) (1: 1,000 in 1% Blotto) for 2 hour. The membrane was then washed with PBS for 4 times with 15 min interval before and reacting with substrate mixture **(Appendix H)** for 5 min and stop reaction with distilled water (DW). The diagram of dot-blotting is shown in **Figure 6.**

Indirect immunofluorescent antibody (IFA)

Preparation of amoeba-fixed slides

Trophozoites of the *E. histolytica* strain HM-1: IMSS were used as antigen for coating the slides. Amoebae from 72 hours culture were chilled for 10 minute and centrifuged at 1,500 rpm for 5 min at room temperatures. Sediment cells were washed three times with NSS and centrifuged at 1,500 rpm for 5 min each. The cells were re-suspended in NSS and spotted onto clean and dry microscopic slides and air-dried on a plate warmer at 37^0C for 30 min. Slides were fixed in cold absolute ethanol in the coupling jar for 10 min at room temperature. The dried slides were wrapped by tissue paper. They were then put in polyethylene bag containing dehydrating substance, sealed and stored at -20^0C until used.

IFA staining for screening of MAb

The technique was performed as described by Thammapalerd *et al*., 1990. Axenic trophozoites of *E. histolytica* strain HM-1: IMSS were used as antigens for coating the slides. The antigen-coated slide was marked into 12 separating square areas by using permanent markers. Each area was placed with 5 µl of Eh208C2-2 MAb and PBS was used as negative control. The slide was left in a humidified plastic box at 37^{o}C for 2 hours and subsequently blotted individually with tissue paper. Its was washed by immersion three times for 3 min each in Coupling jar containing 0.05% Tween 20 (PBST) on rotating apparatus, rinsed again with cold PBST and air dried. A total of 80 µl of fluorescein isothiocyanate (FICT) labeled rabbit anti-mouse IgG conjugate dilution in PBST (1: 20) was added on the slide to cover the whole reacted areas. It was incubated again in a humidified plastic box at 37^{o}C for 1 hour. The washing procedures were repeated and the slide was stored in the dark at 4^{o}C until examination **(Appendix C)**. The result was observed under a fluorescence microscope. The intensity of fluorescence was judged visually and recorded on an arbitrary scale from negative, in which there is no detectable sign of green fluorescence; the areas where the monoclonal antibody was applied gave the strong positive green fluorescence and scored as 4+ **(Appendix D).**

3.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under the non-reducing condition and Western blotting assay

Whole cell lysate (25 µl antigen, 0.1 mg protein /lane) was electrophoresed in comparison with marker proteins of known molecular weights in polyacrylamide gels. One dimentional standard slab SDS-PAGE was used and relative molecular mass was assigned by measuring their relative mobilities in the gels. Stacking gels of 4% and separating gels of 7.5% were used. All reagents used were prepared as described in **Appendix E**.

3.4.1 Preparation and running of the gel

To prepare the running gel, a separating gel was poured between two glass plates (slab gel apparatus; Bio-Rad Laboratories, U.S.A.) to give a 5.8 cm height. The gel was overlaid with distilled water. After 15 min of polymerization at room temperature, all liquid and unpolymerized substances were removed by using Pasteur pipette. The comb was inserted between glass plates , the stacking gel was carefully poured and left for 45 min at room temperature. The comb was gradually removed. The slab gel apparatus was then placed into electrophoretic chamber (mini Protein II electrophoresis apparatus from Bio-Rad) and filled with electrode buffer. The amoebic protein antigens were treated with sample buffer without reducing agent (the mercaptoethanol). The 5 µl antigen extract of *E. histolytica* and the high molecular weight markers were loaded and electrophoresed into the stacking gel slot. Electrophoresis was initially carried out at 120 V per slab gel until the tracking dye reached the separating gel. The current is then decreased to 100 V until the tracking dye was about 10 mm from the bottom edge of the separating gel (Sosa, 1994). The gel was removed from the chamber and then visualized by staining with 0.1 % Coomassie brilliant blue R250 **(Appendix E)**.

3.4.2 Staining and destining of the separated proteins

The separated proteins were stained with 0.1 % Coomassie brilliant blue R250. The reagents and procedure for preparing the solution used are described in **Appendix E**. The gel slab containing SDS-PAGE separated proteins was placed in the 0.1 % Coomassie brilliant blue R250 staining solution at room temperature for 4-8 hours on rocker platform. The stain was removed and the gel was de-stained until the background color was adequately reduced by frequently changing the de-staining solution. When de-staining was completed, the gel was dried, wrapped with cellophane and photo taken.

3.4.3 Molecular Weight determination

The protein antigens and standard proteins of known molecular weights (MW) were concurrently run on the same slab gel. The MWs of protein antigens were calculated by measuring R_f values. R_f value is the ratio of the distance from the top of the gel to each standard MW peptide divided by the distance from the top of the gel to the dye front, a standard curve could be generated. The curve will show the R_f of known MW standard proteins and the log of their MWs. Characterization of the MW protein antigen read directly from the standard curve (please refer to **Appendix F** for the molecular weight standards used).

3.5 Western blotting assay

Electrophoretically separated protein antigens (20 μg protein/land) were transferred onto nitrocellulose (NC) paper using the Transblot apparatus (Bio-Rad) at 80 V for 3 hours in transfer buffer **(Appendix G)**. The nitrocellulose sheet was separated from the gel and quenched in 5% Blotto. Before being done the experiment, the membrane was cut into strips and each strip was incubated with 70 patients' sera separately (group I-III) at 1:200 dilutions. Eh208C2-2 MAb (positive control), as the primary antibody was diluted to 1: 10 in 1% Blotto and incubated for 3 hour at 37^{o}C with nitrocellulose strips. After being extensively washed with 1% Blotto for 4 times with 15 min interval, a secondary antibody with goat anti-human immunoglobulin-horseradish peroxidase conjugate for human sera or goat anti-mouse immunoglobulin-horseradish peroxidase conjugate for mouse MAb were added at a 1:1000 dilution in 1% Blotto, and incubated for 3 hours at 37^{o}C. After being extensively washed, the NC was placed in a freshly prepared substrate solution containing 0.03% DAB, 0.006% H_2O_2, 0.05% $CoCl_2$ in PBS, until the protein bands appeared. NC was washed with DW until the background was clear **(Appendix H).** The diagram of the western blotting assay is described in **Figure 7** employed the 2x2 table as the Mc Nemar's to determine the sensitivity, specificity and efficacy as describe in **Appendix I**

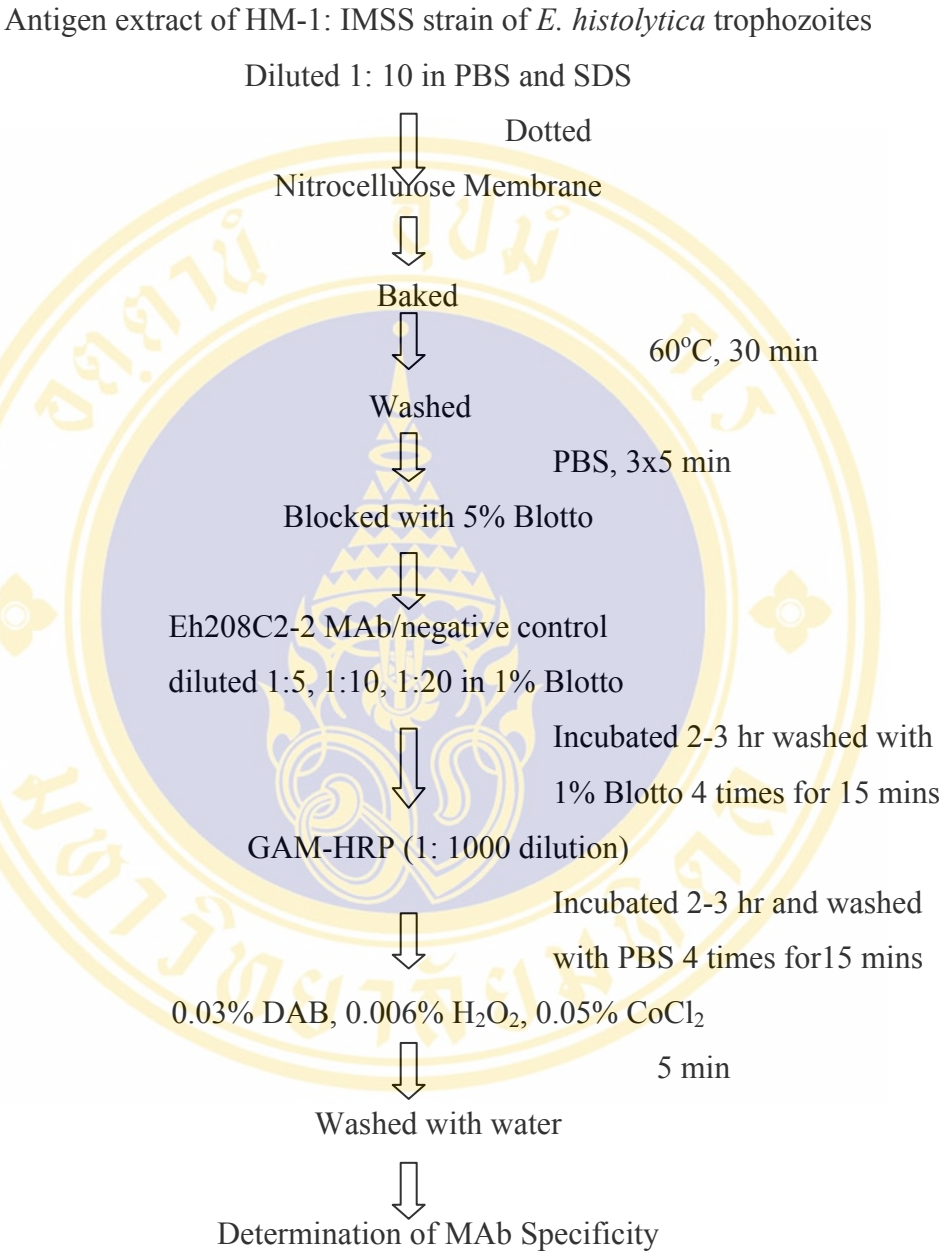


Figure 6. Diagram of Dot-blotting for re-testing the specificity of Eh208C2-2 MAb. Antigen extract of HM-1: IMSS strain of *E. histolytica* tr Antigen extract of HM-1: IMSS strain of *E. histolytica* trophozoites

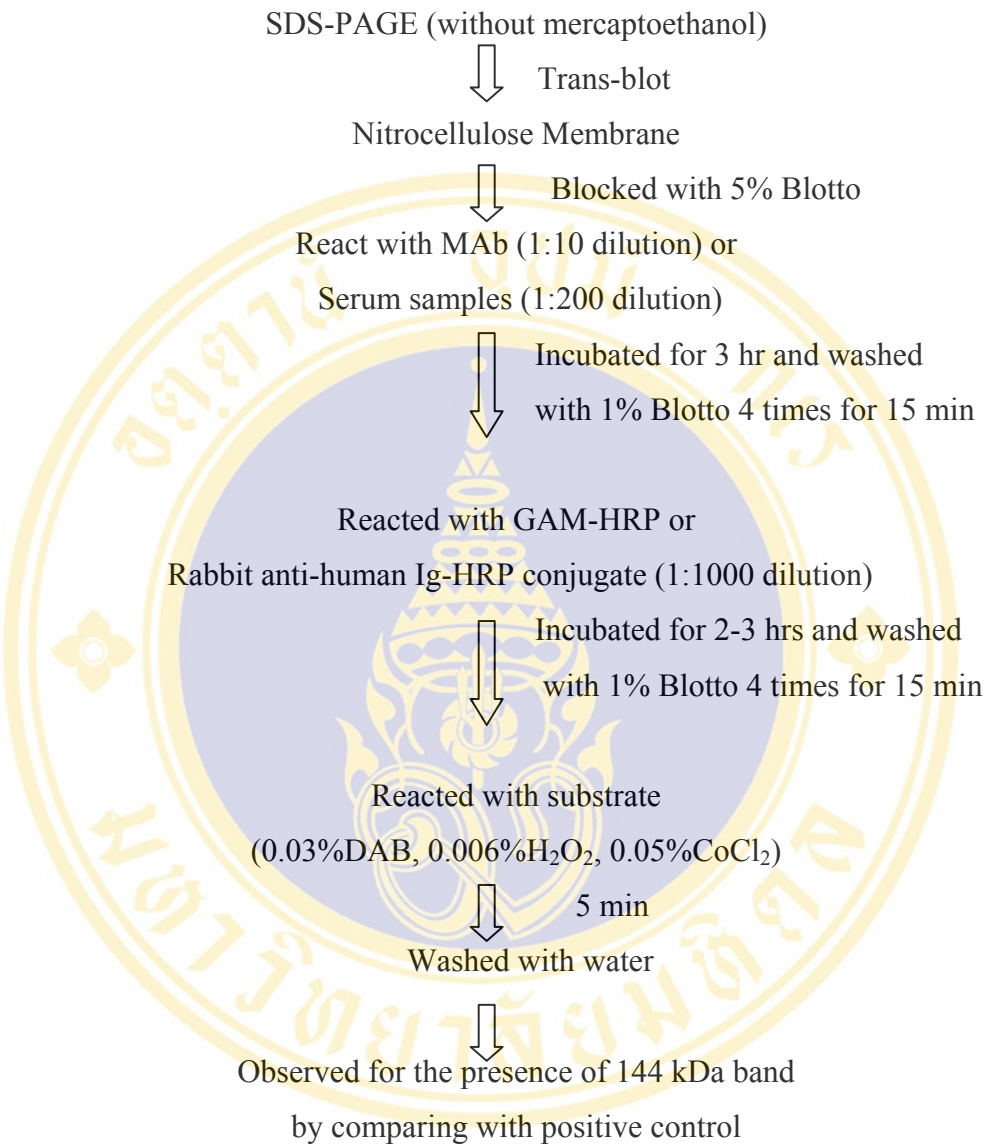


Figure 7. Diagram of Western blotting analysis for characterization of monoclonal antibodies and serum samples.

Table 1. Categories of patients' sera used in this study.

No. of Groups	No. of cases
I Cases of intestinal amoebiasis;	**50**
A. Positive for serological testing by	30
i) Indirect hemagglutination	2
ii) Immunoelectrophoresis	28
B. Positive for *E. histolytica* and/or *E. dispar.* in stools by microscopic examination	30
II Patients have infected with other than *E. histolytica*	**10**
Negative for *E. histolytica* and/or *E. dispar* in stools by microscopic examination	4
i) Positive for *E. coli*	4
ii) Positive for *B. hominis*	2
iii) Positive for *E.* nana	
III Healthy individuals whose stool samples were parasite negative by microscopic examination and serological testing.	**10**
Total	**70**

CHAPTER 4
RESULTS

4.1 Dot-blotting for screening Eh208C2-2 MAb

Dot-blotting is shown in **Figure 8**, the negative control that utilized NSS to replace MAb, displayed pale-white color dot. The whole cell lysate of *E. histolytica* was used as antigen. The antigen diluted in buffer containing PBS gave positive result better than that of SDS. The Eh208C2-2 MAb 1: 5 dilution gave the best results with strongly positive black color dot.

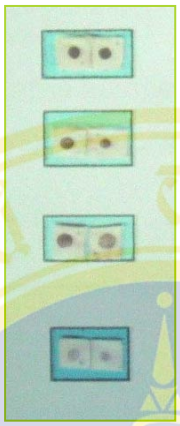
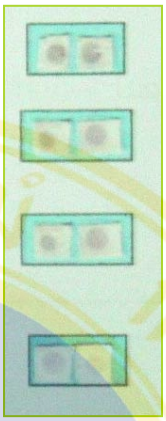
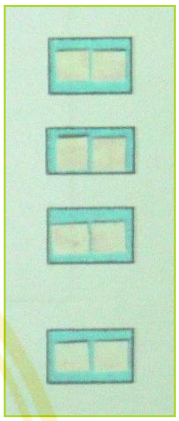
Eh208C2-2 MAb	*E. histolytica* dilute 1: 10 in PBS	*E. histolytica* dilute 1: 10 in 2% SDS	Negative control
Undiluted 1: 5 1: 10 1: 20			

Figure 8. The screening result of Eh208C2-2 MAb by Dot-blotting, *E. histolytica* trophozoites strain HM-1: IMSS was used as antigen.

4.2 Indirect immunofluorescence assay (IFA) for screening Eh208C2-2 MAb

The results of screening for reactivity of Eh208C2-2 MAb are shown in **Figure 9**. Indirect immunofluorescence assay (IFA) was used to detect antibody against *E. histolytica* and FICT-labeled rabbit anti-mouse IgG conjugate was used. NSS was used in placed of Eh208C2-2 MAb as negative control. The positive IFA displayed the bright fluorescence **(Figure 9A)** while negative results showed no detectable sign of fluorescence **(Figure 9B)**.

Figure 9. *E. histolytica* trophozoites strain HM-1: IMSS (size of trophozoite antigens about 8-30 μm) were used for screening sensitivity for Eh208C2-2 MAb by Indirect immunofluorescence assay.

4.3 Protein fraction of *E. histolytica* crude extracts protein on SDS-PAGE under non-reducing condition

Crude extracts of *E. histolytica* trophozoites strain HM-1: IMSS composed of several protein fractions, and separated by 7.5% seperating gel and 4% stacking gel **(Figure 10)**. The Coomassie blue stain displayed several bands of protein fractions approximately 223 to 31 kDa range. The major fractions of peptide bands were 223, 144, 138, 104.7, 97.7, 93, 85, 74, 56, 54, 50, 41, 6, 37 and 31 kDa, and thick bands around 97.7, 74, 54, 50, 41, 6, 37 and 31 kDa. Standard molecular weights proteins were run simultaneously with crude antigen extracts. Based on relative mobility in the gel compared to relative mobilities of known 6 standard molecular weights, the standard calibration curve for the determination of relative molecular weight was constructed (**Figure 11).** (Myosin MW 205,000 kDa; β-Galactosidase MW 116,000 kDa; Phospholipase B MW 97,000 kDa; Albumin MW 66,000 kDa; Oval-bumin MW 45,000 kDa; Carbonic anhydrase MW 29,000 kDa).

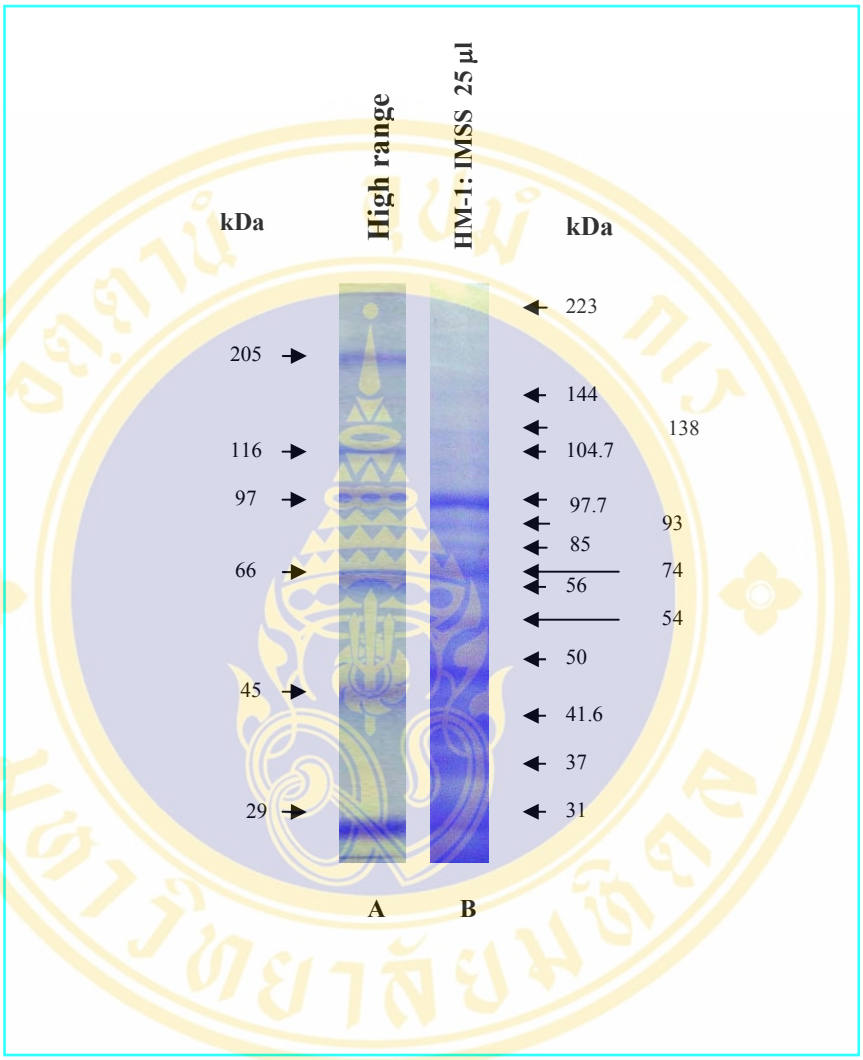


Figure 10. Protein fraction of *E. histolytica* trophozoites strain HM-1: IMSS 25 µl (0.1 mg protein/lane) with separation on 4% stacking gel and separating 7.5% SDS-PAGE under non-reducing condition: (A) High range molecular weights; (B) HM-1: IMSS antigen.

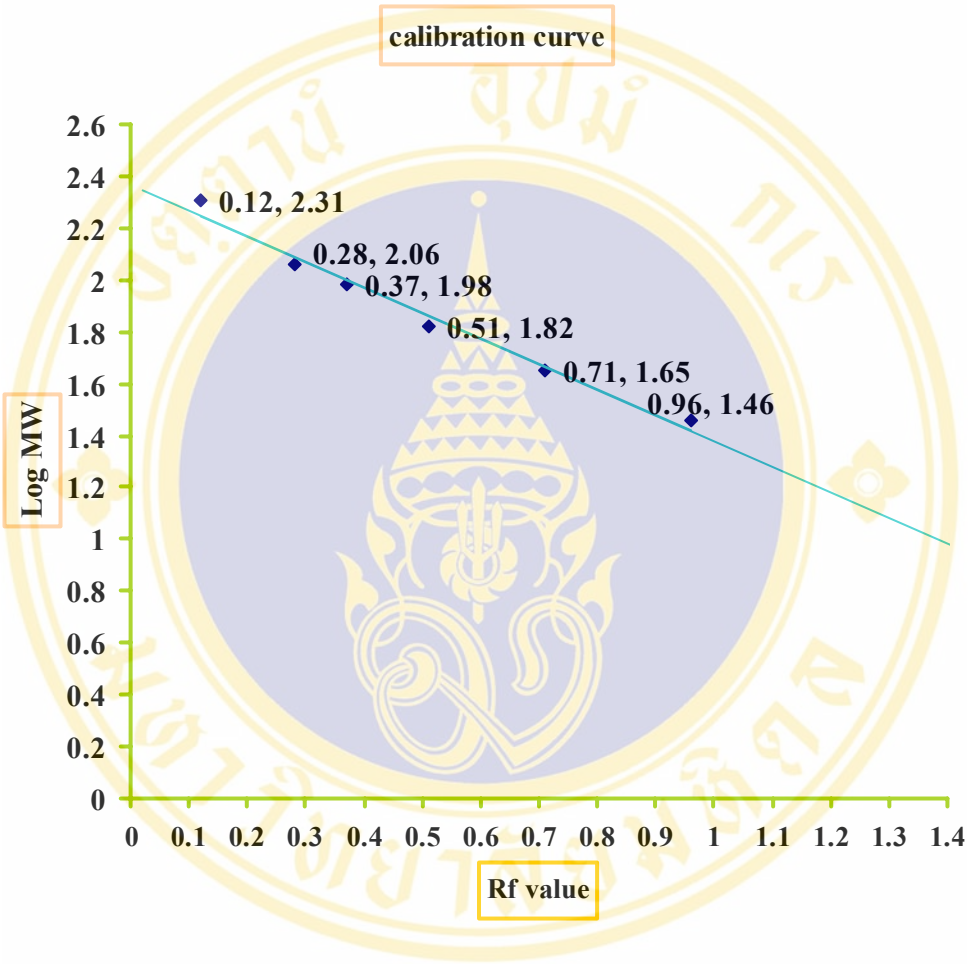


Figure 11. Standard calibration curve for the determination of relative molecular weight of *E. histolytica* HM-1: IMSS trophozoite antigens by SDS-PAGE under non-reducing condition.

4.4 Western blotting

The reaction of Eh208C2-2 MAb when detected with western blotting assay found a single band of anti-PFOR antibody well defined anti-amoebic antibody of molecular weight 144 kDa. The sera from 30 cases of patients (group IA), 10 cases infected with parasite other than *E. histolytica* (group II), 10 cases healthy control sera (group III) and 20 sera positive for *E. histolytica* determined by microscopic by examination (group IB) were identified by western blotting. The result of immunoblotting using serum samples group IB showed several recognized bands with 144, 110, 107, 105, 102, 98, 89, 74, 70, 34 and 30 kDa. One of 144 kDa that was similar to that recognized by Eh208C2-2 MAb (positive control) **(Figure 12)**. Ten serum cases infected with parasite other than *E. histolytica* (group II) composed of; 4 cases infected with *E. coli,* 4 cases infected with *B. homonis* and 2 cases infected with *E. nana*. Among patients infected with *B. homonis*, 1 in 4 cases recognized bands of 115, 83 and 59 kDa. Among patients infected with *E. coli*, 1 in 4 cases recognized band of 115 kDa, while sera infected with *E. nana* did not recognized any band **(Figure 14)** which was similar to results as those in healthy control sera (group III) **(Figure 15)**. The reaction of group IB showed several protein bands, and the fraction of anti-PFOR (144 kDa) antibody was also recognized, at frequency of 35% (found 7 in 20 cases). The sera of patients whose stools were positive for amoebae displayed positive protein bands ranged from 223 kDa-34 kDa (223, 151, 144, 110, 97, 89, 87, 66, 64, 49, 45, 36 and 34 kDa) **(Figure13).**

The results from Mc Nemar's test of sensitivity, specificity and its efficacy group IA was 100%, while the sensitivity, specificity and its efficacy of group IB was 35%, 100% and 56.6% respectively.

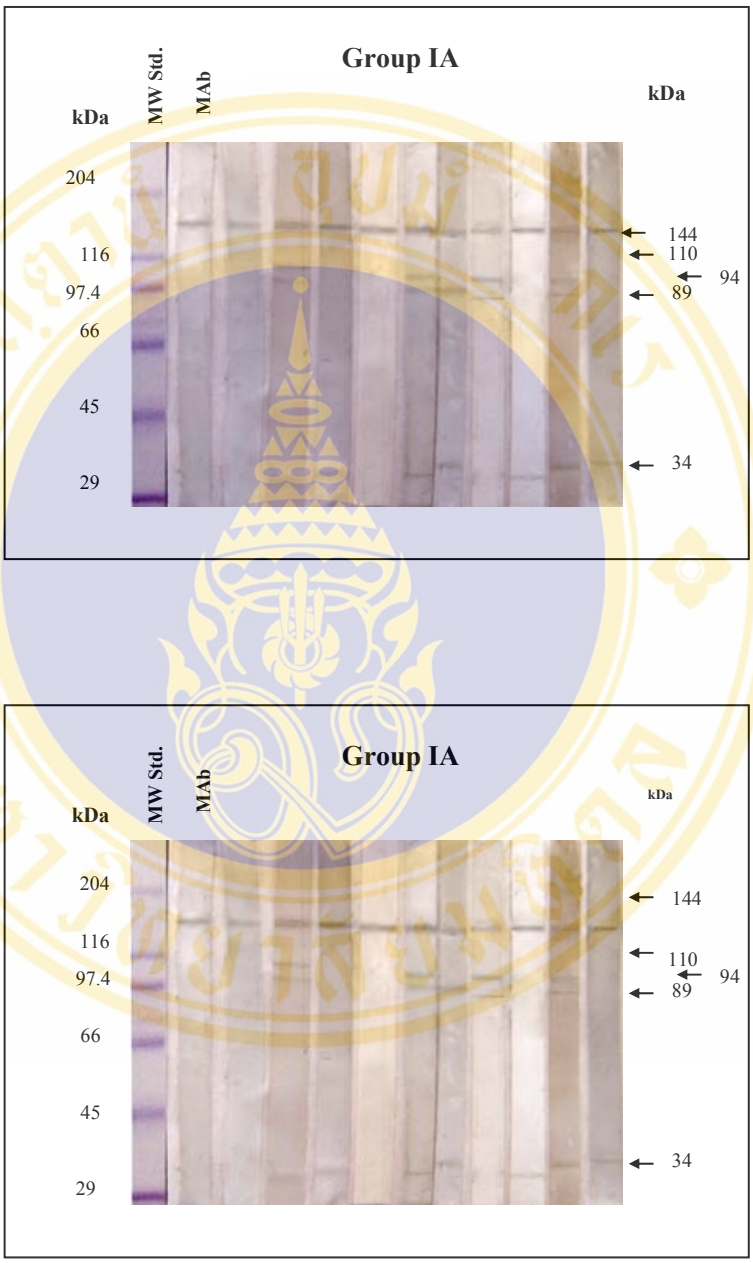


Figure 12. Western blotting of *E. histolytica* HM-1: IMSS trophozoite antigens confronted with sera from patients of group IA by using Eh208C2-2 MAb as positive control.

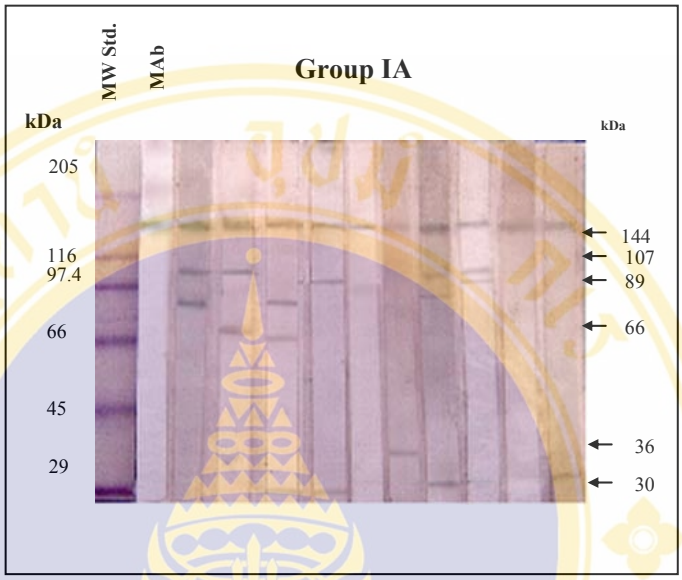


Figure 12 (continued). Western blotting of *E. histolytica* HM-1: IMSS trophozoite antigens confronted with sera from patients of group IA by using Eh208C2-2 MAb as positive control.

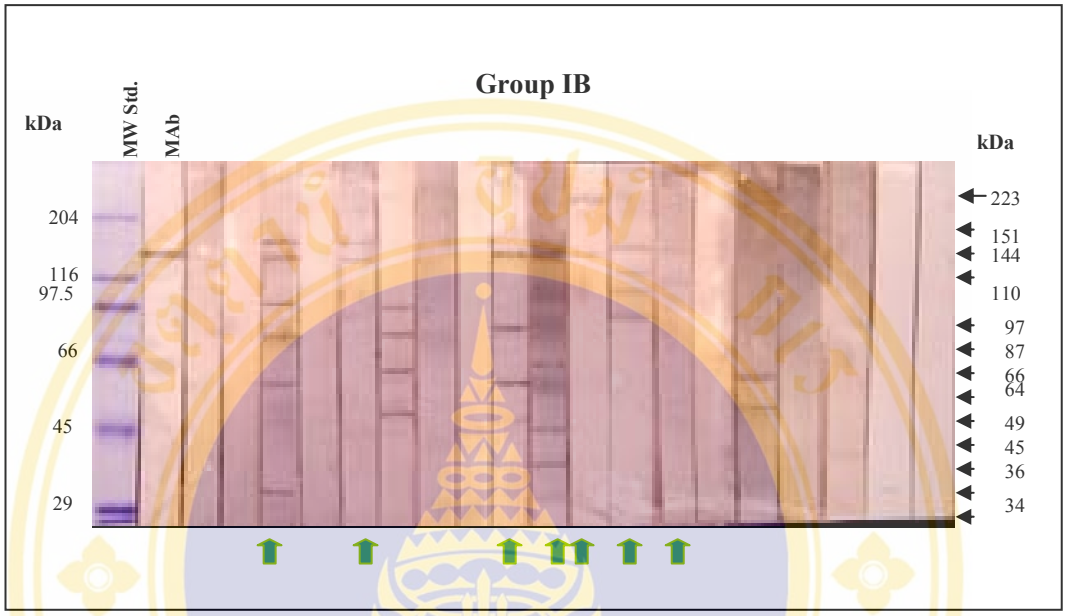


Figure 13. Western blotting of *E. histolytica* HM-1: IMSS trophozoite antigen confronted with sera whose stools were positive for *E. histolytica* as determined by microscopic examination only (group IB). Green arrows showed the presence of anti-PFOR antibody.

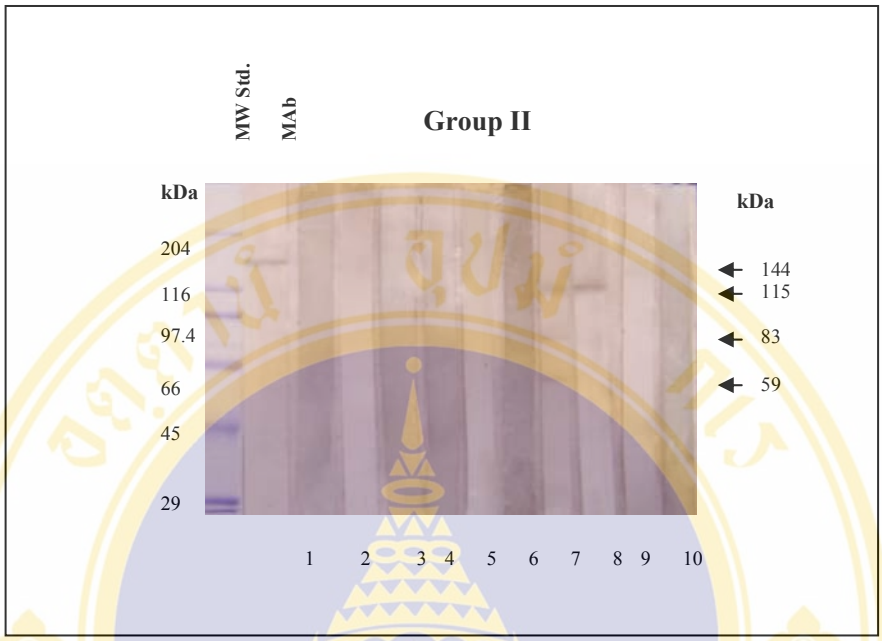


Figure 14. Western blotting of *E. histolytica* HM-1: IMSS trophozoite antigens confronted with serum dilution 1: 200 from: sera infected with parasite other than *E. histolytica* (group II); lane 1-2: *E. nana,* lane 3, 8-10: *E. coli*, lane 4-7: *B. hominis* by using Eh208C2-2 MAb as positive control.

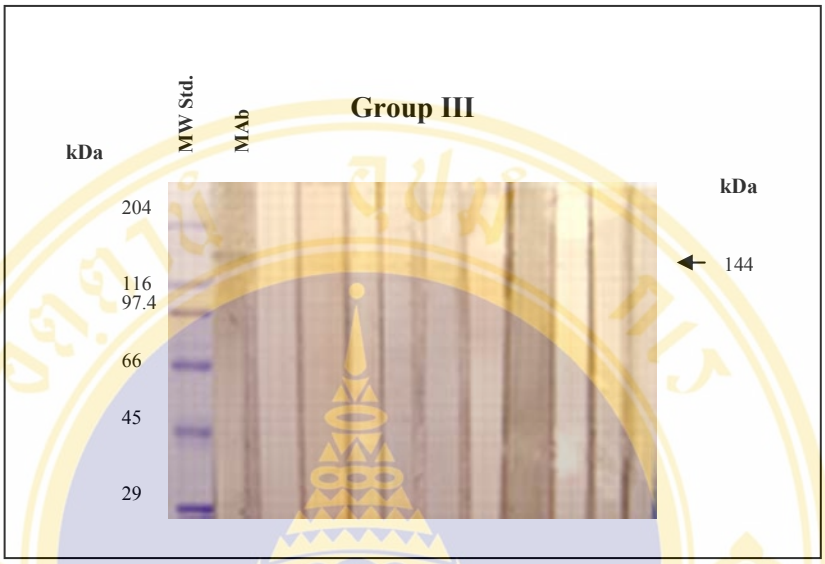


Figure 15. Western blotting of *E. histolytica* HM-1: IMSS trophozoite antigen confronted with serum dilution 1: 200 from healthy control sera (group III) by using Eh208C2-2 MAb as positive control.

Table 3. Categories of patients' sera used in this study and results of Western blotting

No. Groups	No. of cases	Results
I Cases of intestinal amoebiasis;	**50**	**Positive** for anti-PFOR Ab
A. Positive for serological testing by	30	**30 Positive** for anti-PFOR Ab (144 kDa)
i) indirect hemagglutination	2	2
ii) immunoelectrophoresis	28	28
B. Positive for *E. histolytica* in stools by microscopic examination	20	**7 Positive** for anti-PFOR Ab (144 kDa)
II Patients control, have infected with other than *E. histolytica*	**10**	**Negative** for anti-PFOR Ab (144 kDa)
Negative for *E. histolytica* in stool by microscopic examination	4	-
i) positive by *E. coli*	4	-
ii) positive by *B. hominis*	2	-
iii) positive by *E.* nana		
III Healthy individual whose stool sample were negative other parasite in by microscopic examination.	**10**	**Negative** for anti-PFOR Ab (144 kDa)
Total	**70**	**37**

CHAPTER 5
DISCUSSION

Entamoeba histolytica is a pathogen which is world widely prevalent in people who live in poor hygiene. About 10% of infected cases are known to manifest clinical symptoms. This discrepancy is explained by the two species, pathogenic *E. histolytica* and non-pathogenic *E. dispar* (Lee and Tae Hong, 1996). However, the true pathogen may be distinguished if trophozoites containing red blood cells. The assays confirmed by identification of the specific *E. histolytica* stool antigen or by testing blood for specific antibodies (which are not evoked by *E. dispar*) are necessary.

This study involved the developing of an assay to detect anti-pyruvate ferredoxin oxidoreductase (PFOR) antibody in sera of amoebiasis patients by using Western blotting assay. The lyophilized supernatant of Eh208C2-2 MAb was used as positive control. Indirect immunofluorescence assay (IFA) and dot blotting were employed as indicator for immunoreactivity retention of Eh208C2-2 MAb. The positive result by IFA **(Figure 9)** under the blue irradiation of the fluorescent microscope showed consistently greenish-yellow trophozoites with intensity of +4, whereas with the area of negative control, there was no detectable sign of fluorescence. The positive results of dot blotting were obtained **(Figure 8)** suggesting specific binding of antigen diluted in PBS and incubated with Eh208C2-2 MAb at dilution of 1: 5 gave strongly black color dot, while the result of negative control displayed pale-white color.

The crude extracts of *E. histolytica* HM-1: IMSS was characterized by SDS-PAGE under non-reducing condition which revealed band of protein fraction approximately 144 kDa among the other several bands **(Figure 10)**. In addition, Eh208C2-2 MAb reacted to the protein antigen EhPFOR , as determined by western blotting analysis and displayed the band of 144 kDa. It was then concluded that

Eh208C2-2 MAb could react to *Entamoeba* protein antigen corresponding to the molecular weight of 144 kDa.

All patients sera (group IA) recognized 144 kDa with frequencies of 100% **(Figure 12)** which correlated well with PFOR band that reacted with Eh208C2-2 MAb. Sera infected with parasite other *E. histolytica* (group II) **(Figure 14)** and sera from healthy human sera (group III) **(Figure 15)** were examined and showed that they did not recognize the band of 144 kDa. However, patient's sera infected with parasite other *E. histolytica* was used as negative control. 1 out of 4 cases of patients infected with *B. hominis* recognized bands of approximate 115, 83 and 59 kDa, while 1 out of 4 cases patients infected with *E. coli* recognized bands of approximate 115 kDa. The result of sera from patients whose stool were positive for *E. histolytica* (group IB) **(Figure 13)** recognized 144 kDa only 7 out of 20 cases (35%), while 100% of the group IA patients recognized this protein band.

An amoebic PFOR protein was antigen associated with the pathogenicity and triggered an immune response in human and in experimental animals. The anti-PFOR antibody that specific to the pathogenic amoeba have been identified. The sera of asymptomatic patients (group II; 13 cases out of 20 cases), who were infected with non-pathogenic amoeba (*E. dispar*), healthy human sera (group III) and sera infected other *E. histolytica* could not recognize anti-amoebic PFOR antibody.

Using the Mc Nemar's test to compare Microscopy method or serological tests (two gold standards) with Western blotting assay, the sensitivity, specificity and the efficacy of group IA was all 100%, while the sensitivity, specificity and the efficacy of group IB was 35%, 100% and 56.6% respectively.

Diagnostic and therapeutic applications of monoclonal antibodies (MAbs) against *E. histolytica*, one particular, designated Eh208C2-2 MAb have been clearly shown (Thammapalerd and Tharavanij, 1991; Wonsit *et al*., 1992; Scherchand *et al.,* 1994; Sosa *et al.,* 1994). The anti-PFOR antibody was prevalent among the sera of patients with amoebiasis because PFOR is an abundant protein antigen of *E.*

histolytica that was highly conserved, and was immunogenic to the human host suffering from amoebiasis patients (Thammapalerd *et a*l., 1996a). In 1996, Thammapalerd *et al* cloned and sequenced mouse monoclonal antibody, Eh208C2-2MAb recognized specifically and it the amitochondrate *E. histolytica* PFOR (Thammapalerd *et al.,* 1996a), the key enzyme responsible for the decarboxylation of pyruvate and energy production (Kerscher and Oesterhelt, 1982). PFOR was found distributed within trophozoites as well as membrane associated (Sosa *et al.,* 1994; Samarawickrema *et al.,* 1997). An immunotoxin (IT) comprising Eh208C2-2 MAb and the deglycosyllated toxin moiety of ricin A (RA) chain, was studied *in vitro* and *in vivo* and suggested that IT produced was effective against *E. histolytica* (Sosa *et al.,* 1994).

The previous data from immunoblotting studied by Samarawickrema *et al.,* 1997 indicated that *E. histolytica* PFOR is approximately 140 kDa, that consistent with the size of EhPFOR protein (Genbank accession number L46793, Thammapalerd *et al.,* 1996a), while PFOR that predicted by Rodriguez *et al.,* 1998 was 130 kDa. Data from SDS-PAGE of Eh208 C2-2MAb under non-reducing condition indicated that anti-EhPFOR MAb recognized the duplex bands protein of molecular weight 125 kDa and 140 kDa (Sosa, 1994). These data together with previous IFA data (Thammapalerd and Tharavanij, 1991) suggest that PFOR is a membrane associated at some stage probably appear on the parasites surface and cytosol (Rodriguez *et al*., 1998). PFOR in the protozoa *Giadia* and *Trichomonas* is also membrane associated (Hrdy and Muller, 1995).

Western blot procedure could identify the specific and immunogenic fraction of anti-PFOR antibody which should be appeared only in patients' sera, and relevant to PFOR protein recognized by Eh208C2-2 MAb. The high sensitivity, specificity and efficacy in invasive amoebiasis was the advantage of this assay. These evidence indicate that, the method could be developed and applied as routine diagnosis of patients with invasive amoebiasis.

CHAPTER 6
CONCLUSION

Pyruvate: ferredoxin oxidoreductase (PFOR) enzyme has been detected in *Entamoeba histolytica*. It is an abundant and highly conserved immunogenic protein indicating the usefulness in immunodiagnosis of amoebiasis. The outcome of this study involved the identification of 144 kDa band of anti-PFOR antibody against EhPFOR specific parasite antigens among amoebiasis patients (Thammapalerd *et al.,* 1996a).

This thesis study used SDS-PAGE which allowed separation of antigenic fractions of *E. histolytica* antigens and followed by the western blot procedure with sera allowing the identification of specific and immunogenic fraction of anti-PFOR which appear in patients' sera, while Eh208C2-2 MAb, specific to EhPFOR was used as control for the specific protein band characterization at 144 kDa.

Seventy sera used in this study were divided into three groups. 30 Group IA patients sera whose stools were positive for *E. histolytica* as well as by serological tests. 20 group IB sera whose stools were positive for *E. histolytica* as determined by microscopic examination only. 10 Group II sera whose stools were negative for *E. histolytica* but contained other intestinal parasite which were *E. coli, E. nana* and *B. hominis*. 10 Group III uninfected controls whose stools were free from any intestinal parasites. 7 out of 20 sera in group IA recognized 144 kDa whereas sera in group IB recognized 144 kDa which correlated to PFOR band that reacted with Eh208C2-2 MAb. All 10 sera of group II did not recognize 144 kDa; 2 sera infected with *E. nana* were negative. However, 1 out of 4 sera infected with *B. hominis* or with *E. coli* recognized 115, 83, 59 kDa or 115 kDa, respectively whereas all 10 sera of group III were negative. The Western blotting to detect anti-amoebic PFOR antibody could then be one potent immunodiagnosis of invasive amoebiasis.

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APPENDIX

APPENDIX A
CULTURE MEDIA

1. TYI-S-33 (Trypicase, yeast extract, iron-serum) (Diamond *et al.,* 1978)

1.1 TYI broth

Dissolve the following reagents in 50 ml of glass distilled water:

Trypicase (BBL)	2.0 g
Yeast extract (BBL)	1.0 g
Glucose	1.0 g
NaCl	0.2 g
K_2HPO_4	0.1 g
KH_2PO_4	0.06 g
L-cysteine hydrochloride	0.1 g
L-ascorbic acid	0.02 g
Ferric ammonium citrate	2.28 mg

- Bring the final volume to 87 ml with distilled water.
- Adjust pH to 6.8 with 1 N NaOH.
- Clarify by passage through one layer of Whatman No. 1 filter paper.
- Distribute in approximately 10 ml portion to 16 x 125 mm screw-capped test tubes.
- Autoclave 15 min 121^{o}C, 15 lb.
- Cool to room temperature and keep in refrigerator

1.2 Vitamin-Tween 80 mixture

1.2.1 Stock solutions

Solution A : Vitamin mixture 107 (Diamon,1968)

Water soluble B vitamins	500 ml
Biotin solution	250 ml
Folic Acid	250 ml
Lipid soluble vitamins A, D, K	2500 ml
Vitamin E	250 ml

Solution B : Vitamin B12 (Sigma Chem. Co., U.S.A)

- Dissolve 40 mg in distilled water bringing final volume to 100 ml

Solution C : DL-6, 8-Thioctic Acid (Sigma chem. Co., USA.)

- Dissolve 100 mg in absolute ethanol bringing final volume to 200 ml.

Solution D : Tween 80 (Sigma Chem. Co., USA.)

- Dissolve 50 mg in absolute ethanol bringing final volume to 100 ml.

1.2.2 Working solution

Combine the following :

Solution A	100 ml
Solution B	12 ml
Solution C	4 ml
Solution D	4 ml
Distilled water	180 ml

- Sterilize by passage through 0.2 μm Millipore filter.
- Dispense in convenient portions.
- Store in dark at -20^{o}C.
-

1.3 Bovine serum

- Filter sterilize and inactivate for 30 min at 56^{o}C before use.

1.4 Complete medium

- This medium is used for subculturing and maintenance of *E. histolytica.*
- Approximately 10 ml of TYI broth, aseptically add 0.3 ml of Vitamin-Tween 80 mixture and 1.0 ml of bovine serum. Store in dark at -20^{o}C until used.

APPENDIX B
PROTEIN DETERMINATION

Reagent

1. Bradford reagent: Dissolve 100 mg Coomassie Brilliant Blue G-250 in 50 ml 95% Ehanol, add 100 ml 85% (w/v) phosphoric acid. Dilute to 1 liter when the dye has completely dissolved, and filter through Whatman #1 paper just before use.
2. (Optional) 1 M NaOH (to be used if samples are not readily soluble in the color reagent).

Assay

- The Standard Stock Solution of BSA (Bovine Serum Albumin) is 1 mg/mL
- The Stock Solution is Diluted 1:5 with water to 0.2, 0.4, 0.6, 0.8 and 1 mg/mL.
- Add 5 µl of sample/standard into cuvettes.
- Add 250 µl diluted dye (1:5) into each standard/sample well.
- Wait 5 minutes. Read at 595 nM in microplate reader.

Analysis

Prepare a standard curve of absorbance versus micrograms protein, and determine amounts from the curve. Determine concentrations of original samples from the amount protein, volume/sample, and dilution factor, if any. If you are unfamiliar with how to obtain a protein concentration for a diluted sample from a standard curve**.**

APPENDIX C
INDIRECT IMMUNOFLUORESCENCE ANTIBODY ASSAY (IFA)

1. Phosphate Buffered Saline (PBS), pH 7.2, 0.15 M

NaCl	8.00 g
KCl	0.20 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.20 g
Distilled water added to	1000.0 ml
Adjust pH to 7.2	

2. Phosphate Buffered Saline (PBS), pH 7.2, containing 0.05% Tween 20 (PBST)

To prepare 100 ml of the solution, add 0.05 ml of Tween 20.

3. Fluorescein labeled immunoglobulin conjugates

Commercially available fluorescein-labled rabbit anti-mouse immunoglobulin is diluted to 1:20 with PBST before used.

APPENDIX D

CLASSIFICATION OF ANTI– *E. histolytica* MAb BASE ON IFA STAINING PATTERNS

(Thammapalerd and Tharavanij, 1991)

Groups	Staining Pattern
I	Generalized staining of amoebae including the granules, cytosol, internal membrane, and with or without released products
II	Cytoplasmic granules
III	Cytosol
IV	Membrane and cytosol
V	Granules and cytosol

APPENDIX E
SODIUM DODECYL SULPHATE – POLYACRLAMIDE GEL ELECTROPHORESIS

Stock Solutions (All solutions should be filtered.)

1. Monomer solution (30%T 2.7%C_{Bis})

Acrylamide	58.4	g
Bis	1.60	g
H2O	to 200	ml

Store at 4°C in the dark.

2. 4X Running gel buffer (1.5M Tris-HCl, pH 8.8)

Tris	36.3	g

Adjust to pH 8.8 with HCl.

H_2O	to 200	ml

3. 4X Stacking Gel buffer (0.5M Tris-Cl pH 6.8)

Tris	30.0	g

Adjust to pH 6.8 with HCl.

H_2O	to 50.0	ml

4. 10% SDS

SDS		50	g
H_2O	to	500	ml

5. Initiator (10% ammonium persulfate).

ammonium persulfate		0.5	g
H_2O	to	5.0	ml

6. Running gel overlay (0.375 M Tris-Cl pH 8.8, 0.1% SDS).

Tris		25.0	ml Solution (2)
SDS		1.0	ml solution (4)
H_2O	to	100.0	ml

7. Sample Buffer (non-reducing buffer: 6.25 M Tris-HCl pH 6.8, 2% SDS, 10% glycerol).

0.5 M Tris-HCl, pH 6.8	1.0	ml
SDS	1.6	ml Solution(4)
Glycerol	0.8	ml
Distill water	4.0	ml
0.05% (w/v bromphenol blue in water)	0.2	ml

Divide in aliquots and freeze.

Dilute the sample at least 1:4 with sample buffer. Heat at 95^{o}C for 4 minutes.

8. Gel Preparation

8.1 7.5% Seperating gel

30%T 27%C_{Bis}	2.5	ml Solution (1)
1.5M Tris-HCl, pH 8.8	2.5	ml Solution (2)
10% SDS	0.1	ml Solution (4)
DW	4.85	ml

- Mix the solution and gently and degas under vacuum for 10 min.
- Initiate polymerization by adding 50 µl of the 10% ammonium persulfate (freshly prepared) and 3.5 µl of TEMED.
- Pour the gel into the casting apparatus and overlay with distill water.

8.2 4% Stacking gel

30%T 27%C_{Bis}	1.33	ml Solution (1)
0.5M Tris-Cl pH 6.8	2.5	ml Solution (3)
10% SDS	0.1	ml Solution (4)
DW	6.1	ml

- Mix all ingradients gantly, degas under vacuum for 10 min.
- Add 50□l of the 10% ammonium persulfate (freshly prepared) and 5 µl of TEMED.

9. Tank Buffer (0.025 M tris pH 8.3, 0192 M glycine, 0.1 %SDS)

Tris		12.0	ml Solution(3)
Glycine		57.6	g
SDS		40	ml solution
H_2O	to	4.0	ml

10. Stain stock (1% coomassie blue R-250)

Coomassie blue R-250		2.0	g
H_2O	to	200.0	ml
Steir and filter			

11. Stain

(0.125% Coomassie blue R-250, 50% methanol, 10% acetic acid)

Coomassie blue R-250		62.5	ml stain stock (9)
Methanol		250.0	ml
Acetic acid		50.0	ml
H_2O	to	500.0	ml

12. Destaining Solution (50% Methanol, 10% acetic acid)

Methanol		500.0	ml
Acetic acid		100.0	ml
H_2O	to	1000.0	ml

13. Destaining Solution II (7% acetic acid, 5% Methanol)

Acetic acid		700.0	ml
Methanol		500.0	ml
H_2O	to	10.0	lite

APPENDIX F
STANDARD MOLECULAR WEIGHTS

Proteins Standard	Molecular weights (kDa)
Myosin	205,000
β-Galactosidase	116,000
Phospholipase B	97,000
Albumin	66,000
Oval-bumin	45,000
Carbonic anhydrase	29,000

APPENDIX G
WESTERN BLOT

1. Towbin Buffer: for 1 liter

25 mM Tris	3.03	g
192 mM glycine	14.4	g
10% methanol	100	ml
0.1% SDS	1.0	g

Adjust volume to 1 liter with distill water and Cool to 4°C prior to use.

2. 5% Blotto (Blocking Buffer) store at 4°C

Skim milk	5.0	g
PBS	100.0	ml
1% Merthiolate	1.0	ml
Triton X	0.1	ml

3. 1% Blotto (store at 4°C)

Skim milk	1.0	g
PBS	100.0	ml
1% Merthiolate	1.0	ml
Triton X	0.1	ml

APPENDIX H

SUBSTRATE SOLUTION FOR IMMUNO BLOT

Substrate solution

0.03% DAB	3.0	mg
0.006% H_2O_2	10.0	µl
0.05% $CoCl_2$	0.5	ml
PBS	10.0	ml

5 min of incubation is an average time that modify accordingly to your result.

APPENDIX I

Mc Nemar's Test

Microscopic method or serological diagnosis assay	Western blotting (group IA or IB)		Total
	No. Positive	No. Negative	
No. Positive	Tp	Fn	Tp+Fn
No. Negative	Fp	Tn	Fp+Tn
Total	Tp+Fp	Fn+Tn	Tp+Fp+ Fn+Tn

Tp (True Positive)

Patients' sera sample that gave the positive results with microscopic method or sero-positive and gave the positive results result with Western blotting.

Fp (False Positive)

Patients' sera sample that gave the negative results with microscopic method and sero-negative but gave the positive results result with Western blotting.

Fn (False Negative)

Patients' sera sample that gave the positive results with microscopic method or sero-positive but gave the negative results result with Western blotting.

Tn (True Negative)

Patients' sera sample that gave the negative results with microscopic method and sero-negative and gave the negative results result with Western blotting.

$$\textbf{Sensitivity} = \frac{Tp}{Tp+Fn} \times 100 \qquad \textbf{Specificity} = \frac{Tn}{Fp+Tn} \times 100$$

$$\textbf{Efficacy} = \frac{Tp+Tn}{Tp+Fn+Fp+Tn}$$

BIOGRAPHY

NAME	:	Kanlaya Boonpen
DATE OF BIRTH	:	1 September 1977
PLACE OF BIRTH	:	Pathumthanee , Thailand
INSTITUTION ATTENED	:	2000 B.Sc. (Med. Tech.) Faculty Medical Thecnology Rangsit University
HOME ADDRESS	:	Klongluang, Pathumthanee 12120, Thailand.