FINAL REPORT

RESEARCH UNIVERSITY GRANT

Grant No.: 1001/PPSK/813009

Title: ANALYSIS OF *Entamoeba histolytica* ANTIGENS FOR IMPROVED DIAGNOSIS OF AMOEBIASIS

Name of Research Leader:

DR. LIM BOON HUAT
(School of Health Sciences, USM Health Campus)

Name of Co-Researchers:

1. PROFESSOR (DR) RAHMAH NOORDIN
   (INFORMM, USM Penang)
2. PROFESSOR (DR) LALITHA PATTABHIRAMAN
   (Asian Institute of Medicine, Science and Technology, Kedah)
3. DR ALFONSO OLIVOS GARCIA
   (Department of Experimental Medicine,
   Universidad Nacional Autonoma de Mexico)
4. DR ZEEHAIDA MOHAMED (School of Medical Science, USM)
5. DR RUMAIZI SHAARI (School of Dental Science, USM)

UNIVERSITI SAINS MALAYSIA

RESEARCH UNIVERSITY GRANT
FINAL REPORT
Laporan Akhir
Geran Universiti Penyelidikan

A.	**TITLE OF RESEARCH:** *Tajuk penyelidikan:* **ANALYSIS OF *Entamoeba histolytica* ANTIGENS FOR IMPROVED DIAGNOSIS OF AMOEBIAS**
B.	**DETAILS OF RESEARCHER** / *MAKLUMAT PENYELIDIK*
(i)	**Name of Research Leader :** *Nama Ketua Penyelidik* **DR. LIM BOON HUAT**
	Name of Co-Researcher : *Nama Penyelidik Bersama :* 1. **PROFESSOR (DR) RAHMAH NOORDIN** (INFORMM, USM Penang) 2. **PROFESSOR (DR) LALITHA PATTABHIRAMAN** (Asian Institute of Medicine, Science and Technology, Kedah) 3. **DR ALFONSO OLIVOS GARCIA** (Department of Experimental Medicine, Universidad Nacional Autonoma de Mexico) 4. **DR ZEEHAIDA MOHAMED** (School of Medical Science, USM) 5. **DR RUMAIZI SHAARI** (School of Dental Science, USM)
(ii)	**School/Institute/Centre/Unit :** *Pusat Pengajian /Institut/Pusat/Unit :* **PUSAT PENGAJIAN SAINS KESIHATAN**

C.	**Research Platform (Please check (√) for appropriate box):** *Pelantar Penyelidikan (Sila tanda (√) kotak berkenaan):* [ ] A. **Life Sciences** *Sains Hayat* [ ] B. **Fundamental** *Fundamental* [ ] C. **Engineering & Technology** *Kejuruteraan & Teknologi* [ ] D. **Social Transformation** *Transformasi Sosial* [ ] E. **Information & Communication Technology (ICT)** *Teknologi Maklumat & Komunikasi* [ ] F. **Clinical Sciences** *Sains Klinikal* [√] G. **Biomedical & Health Sciences** *Sains Kesihatan & Bioperubatan*
D.	**Duration of this research :** *Tempoh masa penyelidikan ini :* ***Duration :** 3 years + 6 months (extension) *Tempoh :* **From :** 10th October 2007 **To** : 31st March 2011 *Dari:* *Ke :*

ABSTRACT OF RESEARCH

(An abstract of between 100 and 200 words must be prepared in Bahasa Malaysia and in English. This abstract will be included in the Annual Report of the Research and Innovation Section at a later date as a means of presenting the project findings of the researcher/s to the University and the community at large)

Amoebiasis is one of the three most common fatal parasitic diseases in developing countries. Fatality is primarily due to amoebic liver abscess (ALA), an important cause of space-occupying lesions of liver due to invasion of *Entamoeba histolytica* via the colonic mucosa. This research project was undertaken to establish and maintain an axenic culture of *E. histolytica* in Universiti Sains Malaysia; then utilized it to identify and subsequently characterize a specific *E. histolytica* antigen that has diagnostic value via animal experimentation.

The HM1:IMSS *E. histolytica* axenic strain was established and maintained since March 2009 in Biomedicine Laboratory at School of Health Sciences, Universiti Sains Malaysia, in the customized TY1-S-33 medium. Preservation of the amoeba at -80 °C was successful up to approximately 7 days. As such, the culture is still continuously maintained at 36 °C to sustain the *E. histolytica* project. In the animal study, ALA was experimentally developed in Syrian golden hamster by inoculating *E. histolytica* trophozoites into the portal vein. Cardiac puncture was later performed on the morbid hamsters to obtain the ALA serum samples. Based on Western blot analyses on the ALA sera, the ~77 kDa protein was identified as a potential diagnostic biomarker, as it was recognized by 26/31 (83.87%) hamster ALA serum samples, but not detected by any of the healthy hamster sera. MALDI-TOF mass spectrometry results revealed that the protein has a pI value of 5.91 and the amino acid sequence was similar to acetyl-CoA syntase (NCBI C4LUV9) in *Entamoeba histolytica* database. In addition to delivering the two objectives, other studies related to diagnosis of amoebiasis were performed. For instance, we also reported a rapid staining method to detect *E. histolytica* trophozoites in spiked stool sample using Eosin-Y stain. This microscopy staining technique enabled almost instantaneously detection of the trophozoites as compared to the 2-hour preparation period required by the routine Wheatley Trichrome staining technique. Another additional contribution is the application of specific hamster polyclonal antibody in immunohistochemical staining of *E. histolytica* trophozoites in hamster liver. This approach gave better visualization of the trophozoites than either the routine haematoxylin & eosin stain or the periodic acid Schiff stain.

D.	**Abstrak Penyelidikan**

(Perlu disediakan di antara 100 - 200 perkataan di dalam **Bahasa Malaysia dan juga Bahasa**
Abstrak ini akan dimuatkan dalam Laporan Tahunan Bahagian Penyelidikan & Inovasi sebagai
untuk menyampaikan dapatan projek tuan/puan kepada pihak Universiti & masyarakat luar).

Amoebiasis ialah satu daripada tiga jenis penyakit parasit yang menyebabkan paling banyak ke
negara membangun. Kematian terutamanya disebabkan oleh abses hepar amoeba (ALA), iaitu se
hepar akibat daripada jangkitan *Entamoeba histolytica* yang menembusi mukosa kolon. K
dilaksanakan untuk mewujudkan dan mengekalkan kultur aksenik *E. histolytica* di Universiti Sains
yang dapat diguna untuk eksperimen haiwan bagi mengesan dan menciri satu antigen *E. histolytic*
yang mempunyai nilai diagnosis.

Strain aksenik *E. histolytica* HM1:IMSS telah dan masih dikultur dalam medium TY1-S-33 sejak M
di Makmal Bioperubatan, Pusat Pengajian Sains Kesihatan, Universiti Sains Malaysia,. Ameba ir
disejukbekukan pada -80 °C sehingga ~7 hari sahaja. Berhubung dengan kekangan ini, kultu
terpaksa dikekalkan secara berterusan pada 36 °C untuk memastikan projek *E. histolytica* dapat di
Dalam kajian haiwan, trofozoit *E. histolytica* diinokulasi pada vena portal hamster untuk menghas
di dalam heparnya. Darah diperolehi daripada jantung hamster nazak untuk memperoleh samp
ALA. Menerusi analisis blot Western blot, protein dengan berat molekul ~77 kDa telah dikenal pas
biomarker yang berpotensi diguna dalam diagnosis ALA manusia. Protein ini telah dicam o
(83.87%) sampel serum hamster ALA, tetapi langsung tidak dikesan oleh serum hamster sihat. K
spetktrofotomeri massa MALDI-TOF menunjukkan protein ini mempunyai nilai pI 5.91 dan ur
aminonya adalah sama dengan acetyl-CoA syntase (NCBI C4LUV9) pada pangkalan data *E*
histolytica. Di samping menunaikan dua objektif kajian di atasi, kajian tambahan berhubun
diagnosis amebiasis juga dilaksanakan. Kami telah berjaya memperkenal satu kaedah pewarn
menggunakan pewarna Eosin-Y untuk mengesan trofozoit *E. histolytica* yang berada di dalam sa
Teknik ini membolehkan pemerhatian trofozoit dilaksanakan dengan segera berbanding dengan t
(Wheatley Trichrome) yang mengambil masa hampir dua jam. Satu lagi penemuan tambahan pe
penggunaan antibodi spesifik dalam pewarnaan imunohistokimia terhadap trofozoit *E. histolytic*
hati hamster. Pendekatan ini memberi visualisasi yang lebih jelas berbanding dengan pewa
haematoxylin & eosin atau pewarnaan Schiff asid periodik.

F. SUMMARY OF RESEARCH FINDINGS
Ringkasan Penemuan Projek Penyelidikan

The research has generated:

1. One (ongoing) PhD student.
2. One MSc student is waiting for his viva voce and another has submitted his notic submission.
3. Three undergraduate final-year projects
4. Four published articles in peer-reviewed journals (Total Impact factors: 1.812 + 0.65 + 3.112) and one more is under review at Journal of Vaccine and Clinical Immunology
5. Nine (9) oral and six (6) poster scientific presentations in both national and international
6. A sustainable axenic culture of *E. histolytica* in Biomedicine Laboratory, School Science, USM.
7. One patent filed for the Malaysian Patent
8. One international collaboration with Dr. Alfonso Olivos Garcia (Universidad Auton Mexico) and one national collaboration with Professor (Dr.) Lalitha Patthabiraman (AIMS

G. **COMPREHENSIVE TECHNICAL REPORT**

Applicant are required to prepare a Comprehensive Technical Report explaning the project. (This report must be appended separately)
Sila sediakan laporan teknikal lengkap yang menerangkan keseluruhan projek ini. [Sila gunakan kertas berasingan]

PLEASE REFER TO APPENDIX 1: COMPREHENSIVE TECHNICAL REPORT

List the key words that reflects your research:
Senaraikan kata kunci yang mencerminkan penyelidikan anda:

English	Bahasa Malaysia
Entamoeba histolytica	*Entamoeba histolytica*
Amoebiasis	Amebiasis
Amoebic liver abscess (ALA)	Abses hati amoeba
Hamster	hamster
Esoin-Y stain	Pewarna Eosin-Y
Immunohistochemical (IHC) stain	Pewarna imunohistokimia (IHC)
Western blot	Blot Western
Acetyl-CoA syntase	Acetyl-CoA syntase

H. a) **Results/Benefits from this research**
Hasil Penyelidikan

No. *Bil:*	Category/Number: *Kategori/ Bilangan:*	Promised	Achieved
1.	**Research Publications** (Specify target journals) ***Penerbitan Penyelidikan*** *(Nyatakan sasaran jurnal)*	4	**5 research publications:** (a) 1 in Parasitology Research; (b) 2 in Tropical Biomedicine; (c) 1 in Asia Pacific Journal of Tropical Biomedicine (d) ~~1 still under review~~ Accepted for pn at Journal of Vaccine & Clinical Immunology
2.	**Human Capital Development**		
	a. **PhD Students**	1	**1** (ongoing)
	b. **Master Students**	0	**2** [**1** waiting for viva voce; **1** submit notice for thesis submission]
	c. **Undergraduate Final Year Project**	0	**3** (Completed)
	d. **Research Officer**	1	**1** (Assistant RO)
	e. **Research Assisstant**	0	**1** (RA)
	f. **Others: Please Specify**	0	**1** (Completed an industrial training 1 one MSU student)
3.	**Patents** *Paten*	0	**1** (Filed for patent)
4.	**Specific @ Potential Applications** *Spesifik/Potensi Permohonan*	1	**1** (Recombinant proteins of the poten biomarkers for diagnosis of ALA)
5.	**Networking & Linkages** *Jaringan & Jalinan Kerjasama*	2	**2** (Dr Alfonso OG & Prof. P. Lalitha
6.	**Possible External Research Grants to be Acquired** *Jangkaan Geran Penyelidikan Luar Dipohon*	0	0
7.	**Product/Prototype** *Produk/Prototaip*	0	0

- Kindly provide copies/evidence for Category 1 to 7.

b) Equipments used for this research.
Peralatan yang telah digunakan dalam penyelidikan ini.

Items *Perkara*	Approved Equipment	Approved Additional Equipment	Location
Specialized Equipment Peralatan khusus	1. One Micropipettor 2. One SDS-PAGE electrophoresis 3. One 12-Channel pipettor 4. One Pipette-aid	5. One Refrigerator 6. One -20°C Refrigerator 7. One 37°C Incubator	All equipment are in Biomedicine Laboratory School of Health Scienc USM
Facility Kemudahan	Not applicable	Not applicable	Not applicable
Infrastructure Infrastruktur	Not applicable	Not applicable	Not applicable

Note: Although the hemocytometer was approved in the grant, it was not purchased as it was availab in PPSK.

I. BUDGET / *BAJET*

Perbelanjaan :: Expenditure

Project Account No. : 1001/PPSK/81300

Total Approved Budget : RM 197,476.00
Total Additional Budget : RM 56,003.03
Grand Total Approved Budget : RM 253,479.03

Yearly Budget Distributed
Year 1 : RM 80,660.00
Year 2 : RM 62,158.00
Year 3 : RM 54,658.00

Additional Budget Approved
Year 1 : RM 0
Year 2 : RM 0
Year 3 : RM 56,003.03

Total Expenditure : RM 253,099.13
Balance : RM 379.90

- **Please attach final account statement from Treasury**

UNIVERSITI SAINS MALAYSIA

JABATAN BENDAHARI

KUMPULAN WANG UNIVERSITI PENYELIDIKAN (RU)

PENYATA PERBELANJAAN SEHINGGA 31 JULAI 2011

	RM
Jumlah Geran :	253,479.03
Peruntukan 2007 (Tahun 1)	80,660.00
Peruntukan 2008 (Tahun 2)	62,158.00
Peruntukan 2009 (Tahun 3)	54,658.00
Tambahan 1 (Tahun 3)	34,356.00
Tambahan 2 (Tahun 3)	21,647.03

Ketua Projek : DR LIM BOON HUAT

Tajuk Projek: Analysis Of Entamoeba Histolytica Antigents For Improve Diagnosis Of Emoebiasis

Tempoh : 3 Tahun (10/10/2007-30/09/2010)

T. Lanjut : 01/10/2010-31/03/2011

No. Akaun : 1001/PPSK/813009

Kwgan	Akaun	PTJ	Projek	Peruntukan Projek	Perbelanjaan Terkumpul sehingga Tahun lalu	Peruntukan Semasa	Tanggungan Semasa	Bayaran Tahun Semasa	Belanja Tahun Semasa	Baki Projek
1001	11000	PPSK	813009	72,000.00	23,444.63	48,555.37			-	48,555.37
1001	14000	PPSK	813009			-			-	-
1001	15000	PPSK	813009			-			-	-
1001	21000	PPSK	813009	44,356.00	29,875.52	14,480.48			-	14,480.48
1001	22000	PPSK	813009			-			-	-
1001	23000	PPSK	813009	900.00	3,050.80	(2,150.80)			-	(2,150.80)
1001	24000	PPSK	813009			-			-	-
1001	25000	PPSK	813009			-			-	-
1001	26000	PPSK	813009	3,500.00		3,500.00			-	3,500.00
1001	27000	PPSK	813009	107,423.03	133,567.54	(26,144.51)		7,147.95	7,147.95	(33,292.46)
1001	28000	PPSK	813009			-			-	-
1001	29000	PPSK	813009	3,300.00	30,037.69	(26,737.69)		500.00	500.00	(27,237.69)
1001	32000	PPSK	813009			-			-	-
1001	35000	PPSK	813009	22,000.00	25,475.00	(3,475.00)				

Signature of Researcher
Tandatangan Penyelidik

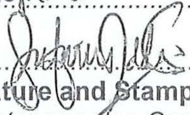
8/9/2011

Date
Tarikh

General Comments:
Ulasan Umum:

H. RESEARCH COMMITTEE COMMENTS
KOMEN JAWATANKUASA PENYELIDIKAN PERINGKAT PTJ

Overall achievement is very good. The research has been completed successfully. Four articles have been published in international journals and 1 patent has been filed.



Signature and Stamp of Chairperson of PTJ's Evaluation Committee
Tandatangan dan Cop Pengerusi Jawatankuasa Penilaian PTJ

Date : 7/9/2011
Tarikh :

PROF.MADYA ZAFARINA ZAINUDDIN
Timbalan Dekan
(Penyelidikan & Pengajian Siswazah)
Pusat Pengajian Sains Kesihatan

PROFESOR DR. AHMAD HJ ZAKARIA
Dekan
Pusat Pengajian Sains Kesihatan
Universiti Sains Malaysia
Kampus Kesihatan
16150 kubang Kerian
Kelantan.

Signature and Stamp of Dean/ Director of PTJ's
Tandatangan dan Cop Dekan/ Pengarah PTJ

Date : 13/9/11
Tarikh :

ANALYSIS OF *Entamoeba histolytica* ANTIGENS FOR IMPROVED DIAGNOSIS OF AMOEBIASIS

By

LIM BOON HUAT[1]
RAHMAH NOORDIN[2]
LALITHA PATTABHIRAMAN[3]
ALFONSO OLIVOS GARCIA[4]
ZEEHAIDA MOHAMED[5]
RUMAIZI SHAARI[6]

[1]School of Health Sciences, [2]USM Health Campus INFORMM, USM Main Campus; [3]Asian Institute of Medicine, Science and Technology, Kedah; [4]Department of Experimental Medicine, Universidad Nacional Autonoma de Mexico; [5]School of Medical Sciences, USM Health Campus; [6]School of Dental Sciences, USM Health Campus

PROJECT FUNDED BY UNIVERSITI SAINS MALAYSIA RESEARCH UNIVERSITY GRANT [1001/PPSK/813009]
10th October 2007 – 31st March 2011

ABSTRACT

Amoebiasis is one of the three most common fatal parasitic diseases in developing countries. Fatality is primarily due to amoebic liver abscess (ALA), an important cause of space-occupying lesions of liver due to invasion of *Entamoeba histolytica* via the colonic mucosa. This research project was undertaken to establish and maintain an axenic culture of *E. histolytica* in Universiti Sains Malaysia; then utilized it to identify and subsequently characterize a specific *E. histolytica* antigen that has diagnostic value via animal experimentation.

The HM1:IMSS *E. histolytica* axenic strain was established and maintained since March 2009 in Biomedicine Laboratory at School of Health Sciences, Universiti Sains Malaysia, in the customized TY1-S-33 medium. Preservation of the amoeba at -80 ^{o}C was successful up to approximately 7 days. As such, the culture is still continuously maintained at 36 ^{o}C to sustain the *E. histolytica* project. In the animal study, ALA was experimentally developed in Syrian golden hamster by inoculating *E. histolytica* trophozoites into the portal vein. Cardiac puncture was later performed on the morbid hamsters to obtain the ALA serum samples. Based on Western blot analyses on the ALA sera, the ~77 kDa protein was identified as a potential diagnostic biomarker, as it was recognized by 26/31 (83.87%) hamster ALA serum samples, but not detected by any of the healthy hamster sera. MALDI-TOF mass spectrometry results revealed that the protein has a pI value of 5.91 and the amino acid sequence was similar to acetyl-CoA syntase (NCBI C4LUV9) in *Entamoeba histolytica* database.

In addition to delivering the two objectives, other studies related to diagnosis of amoebiasis were performed. For instance, we also reported a rapid staining method to

detect *E. histolytica* trophozoites in spiked stool sample using Eosin-Y stain. This microscopy staining technique enabled almost instantaneously detection of the trophozoites as compared to the 2-hour preparation period required by the routine Wheatley Trichrome staining technique. Another additional contribution is the application of specific hamster polyclonal antibody in immunohistochemical staining of *E. histolytica* trophozoites in hamster liver. This approach gave better visualization of the trophozoites than either the routine haematoxylin & eosin stain or the periodic acid Schiff stain.

KEYWORDS: *Entamoeba histolytica*, amoebiasis, amoebic liver abscess (ALA), hamster, Eosin-Y stain, immunohistochemical (IHC) stain, Western blot and acetyl-CoA syntase

*Corresponding author: Tel: +6097619; Fax: +6097677515; limbh493@gmail.com

1. INTRODUCTION

Amoebiasis is a parasitic infection acquired through ingestion of food or water contaminated with cysts of *E. histolytica*. The parasite causes intestinal infection, diarrhoea and extraintestinal amoebiasis. The estimated global burden of the disease in humans is about 50 million with up to 100,000 deaths annually (Tanyuksel & Petri, 2003; Wells & Arguedas, 2004). In Malaysia, 9.9 % of children were infected (Rajeswary et al., 1994) and about 40% of liver abscess cases were found to be amoebic in origin (Goh et al., 1987; Jamaiah & Shekhar, 1999).

Despite its inherent drawbacks, microscopy continues to be the diagnostic method used in most laboratories around the world (Clark, 2004). The existing Wheatley trichrome staining technique is time-consuming as it requires at least 2-hour to prepare, and the stain cannot differentiate *E. histolytica* from non-pathogenic *E. dispar* (Tanyuksel & Petri, 2003). Unfortunately, only ~12% of patients with amoebic liver abscess (ALA) were reported with the amoebas in their stool samples (Wells & Arguedas, 2004). *E. histolytica* trophozoites were reported in up to 85% of liver abscess (Juimo et al., 1992), but aspiration of the abscess material is both invasive and technically demanding (Stanley, 2003). Culture/zymodeme analysis is specific; however one species may outgrow the other in cultures of specimens from mixed infections (Wells & Arguedas, 2004). Molecular techniques are highly sensitive and specific but they require a longer time to perform, technically complex, and also relatively costly (Stanley, 2003; Tanyuksel & Petri, 2003). A promising commercially available noninvasive antigen detection test was reported to detect circulating galactose/N-Acetyl galactosamine-specific lectin (or Gal/GalNac lectin) antigen in sera of 96% (22/23) ALA patients (Jamaiah & Shekhar, 1999). However, in another

study, the test kit detected the antigen in only 2.3% (1/43) of patients suspected of having ALA; although 76.7% (33/43) of them were found to be positive for anti-lectin antibody (Wan Nor Azilah, 2007).

Hence this study aimed to establish an axenic culture of *E. histolytica* in Biomedicine Laboratory of School of Health Sciences, Universiti Sains Malaysia, and subsequently utilizes the amoeba for research to improve on the existing diagnostic tools. Obtaining truly human ALA serum samples for research is difficult, as current diagnosis depends on ultrasonic and serological investigations. Technically, confirmation of ALA should be based on identification of *E. histolytica* trophozoites in abscessed liver biopsy, but this approach is invasive and considered unethical as most trophozoites are found in the healthy liver cells at the peripheral of the liver abscess. Thus, this study also aimed to identify and characterize a specific *E. histolytica* antigen that has diagnostic value via animal experimentation, in which ALA will be developed in susceptible hamster to obtain truly ALA serum samples for Western blot analysis.

2. MATERIALS AND METHODS

2.1 Maintenance of *E. histolytica* trophozoites

2.1(a) Preparation of TYI-S-33 medium for *E. histolytica* culture

To prepare 1 L of TYI-S-33 medium, solutions A and B have to be prior prepared. Solution A was prepared by dissolving 30 g of Biosate peptone (Becton Dickinson, Lot No: 7050570) and 10 g Dextrose in 500 mL of distilled water. For solution B, 1 g of Dipotassium phosphate (K_2HPO_4), 0.6 g of Monopotassium phosphate (KH_2PO_4), 2 g of Sodium chloride (NaCl), 1g of Cysteine and 0.2 g of ascorbic acid were dissolved in 370 mL of distilled water. Solutions A and B were then mixed and subsequently added with 22.5 mg of ferric ammonium citrate. The pH of the solution was adjusted to 6.8 with 2 N NaOH, and filtered (Whatman, Cat. No: 1004240) before it was autoclaved at 121 °C for 10 minutes. When the autoclave temperature dropped to 90 °C, the bottle of solution was taken out and allowed to cool until it reached room temperature. Then, 30 mL of vitamin solution (SAFC Bioscience, Lot No: 7L0326) and 100 mL of decomplemented bovine serum (Invitrogen, Lot No: 667195) were added into the sterilized solution to form the TYI-S-33 medium, which was kept at 4 °C until it was ready to be used.

2.1(b) Preparation of Streptomycin-Benzylpenicillin-Kananmycin Antibiotics

Six gram of streptomycin sulfate and 3.6 g of benzylpenicillin sodium salt were dissolved in 6 mL of distilled water. Then, 2 g of kanamycin was added. The mixture was syringe-filtered through 0.20 μm filter disc (Schleicher & Schuell, Lot No: DR0700-3) and kept in 4 °C.

2.1(c) Viability Testing

First, a 1:1 dilution of amoeba suspension with 0.4% Trypan blue solution was prepared. Next, the mixture was loaded onto a hemocytometer counting chamber. The number of unstained amoebas (viable cells) and stained (non-viable cells) were counted. Finally, the viability of amoeba was determined based on the calculations below:

Total number of viable cells = average number of viable cells x 10^4 x dilution factor

= n x 10^4 x 2

$$\text{Viability of amoeba} = \frac{\text{Total number of viable cells} \times 100\ \%}{\text{Total number of cells}}$$

2.1(d) Hemocytometer Counting

A clean cover-slip was placed on a clean hemocytometer counting chamber. A drop of amoeba suspension was loaded at the edge of the chamber and the suspension was allowed to be drawn into the chamber by capillary action. The chamber was then placed on the microscope stage and the average number of amoeba was determined using the formula below.

Formula: $c = n/v$

Where c = cell suspension in cells/mL; n = average number of cells/mm^2 area and v = volume counted = 10^4 ($1mm^2 = 0.1\ mm^3 = 10^{-4}$mL).

Thus, $c = n \times 10^4$

2.1(e) Harvesting of Amoebic Culture

Prior to culturing of the axenic *E. histolytica*, a viability test was performed. Then, an appropriate amount of the amoeba was transferred into each culture tube containing 7.5 mL of TYI-S-3 medium. The culture tube was placed in a horizontal position 36 °C for 72 hr. (Diamond et al., 1978). The amoebas were harvested by first cooling the culture tubes on ice for 5 minutes. Then, the culture medium were transferred into sterile 1.5 mL microcentrifuge tubes and spun at 440 x g for 3 minutes at 4 °C. After decanting the medium, 1 mL of sterile 1X PBS was added to resuspend the amoeba pellet. The amoeba suspension was again washed by centrifugation. After removing the supernatant, the amoeba pellet was stored at -80 °C.

2.1(f) Maintenance of Virulent *E. histolytica* and Collection of Hamster ALA Serum Samples

To maintain the virulence of axenic *E. histolytica* (HM1-IMSS), each fortnight the amoebas in TYI-S-3 medium were used to develop ALA in a Syrian golden hamster (*Mesocricetus auratus*). The experimental development of ALA in hamster was performed as described by Olivos-Garcia et al. (2004) and Weber et al. (2008). Briefly, 1 x 10^6 trophozoites suspended in 0.2 mL phosphate buffer saline (PBS) was inoculated into the portal vein of an anesthetized (6 mg pentobarbital / 100 g body weight) male hamster. To prevent excessive bleeding, a small piece of gelfoam (Pharmacia & Upjohn Co., USA) was placed onto the injected site of portal vein and held for one minute with a piece of gauze. About a week later, the morbid animal was sacrificed with 3X overdose of pentobarbital. Cardiac puncture was performed on the euthanized animal to collect the hamster ALA serum sample. Following which, the abscessed liver was transferred aseptically onto a sterile Petri dish and cut into small fragments. Some fragments were transferred into a sterile culture tube and filled with 7.5 mL of TYI-S-33 medium followed

by adding 15 μL of streptomycin-benzylpenicillin-kananmycin antibiotics. The culture tube was then incubated at 36°C in a horizontal position. After 24 hours of incubation, the tube wall was observed under inverted microscope to check for amoebas. The medium was replaced with fresh medium and antibiotics. The procedure was repeated each 24 hours for 5 days, in which the antibiotics were gradually reduced. Finally, the amoebas were cultured in the absence of antibiotics and ready to be harvest in TYI-S-33 medium. The animal experimentation above was approved by USM Animal Research Ethics Committee [No. Animal Ethics Approval: USM/Animal Ethics Approval/ 2008/(40)(129)].

2.1(g) Preservation of *E. histolytica*

Preservation of *E. histolytica* was performed as suggested by Samarawickrema et al. (2001) with modifications. Following the DMSO stop, the amoebas was immediately preserved in a -80 °C freezer instead of being first subjected to rapid cooling at -70 °C for 48 hr, followed by cryopreservation in liquid nitrogen.

2.2 Analysis of *E. histolytica* antigens

2.2 (a) SDS-PAGE

Protein samples were electrophoretically separated via SDS-PAGE using Bio-Rad Mini Protean III Electrophoresis Cell and Protean® II xi Cell according to Laemmli (1970) protocol with modifications. Prior to SDS-PAGE, crudes soluble antigen of *E. histolytica* was mixed with 2X Laemmli sample buffer and boiled for 5 min. Subsequently, it was separated using 6% or 10% SDS-PAGE gel, at constant current of 25 mA per gel for about 1 h.

2.2 (b) Western blotting

Upon completion of SDS-PAGE, proteins in the gel was electrophoretically transferred onto a 0.45 μm nitrocellulose membrane (NCP) using semi-dry transblot (Bio Rad, USA) at a constant voltage of 15 V for 30 min. The NCP was blocked for 1hr at RT with 5 % skim milk prepared in 10 mM Tris buffered saline, pH 7.2 (TBS). Subsequently, the NCP was washed (3 x 5 min) with TBS containing 0.1 % Tween-20 (TBS-T). Then, the NCP was cut into multiple strips and incubated with hamster sera at dilution of 1:50 (in TBS-T) for 2 hours at RT. The NCP strips were then washed three times with TBS-T, and then incubated with monoclonal mouse anti–hamster IgG conjugated with horseradish peroxides (HRP) at dilution of 1:4000 for 1hr. Subsequently, the NCP strips were again washed (3 x 5 min) with TBS-T. Western blot substrates *i.e.* enhanced chemiluminescence (ECL) blotting reagent (Roche diagnostics, Germany) or tetramethylbenzidine (TMB) substrate for membrane (Sigma, USA) were used as substrates. The Western blot signal was captured using camera (Lumix, Germany).

2. 3 Rapid Eosin-Y Staining

The Eosin-Y stain (Sigma HT110316, USA) used in this study was in its working dilution, thus was applied directly on stool samples without fuss. Alternatively, Eosin-Y staining solution can be prepared by mixing 1% (w/v) Eosin-Y, 1% (w/v) phloxine-B, 95% ethanol and glacial acetic acid in appropriate volumes (Mayer, 2009). Briefly, approximately one million axenic *E. histolytica* was washed with 1X PBS and spiked in 2 g of fresh semi-solid stool sample obtained from a healthy volunteer. An applicator stick was used to mix ~30 μL of the Eosin-Y stain with ~2 mg of stool sample on a clean slide. DPX was used to seal the cover slip placed over the sample to the slide, which was then observed under a microscope at 400X and 1000X magnification. The images of the stained

trophozoites were captured using Olympus Image Analysis System (Olympus System Microscope Model BX41, Japan).

2.4. Immunohistochemical Staining

Indirect staining was performed on processed tissue sections with some modifications on the standard protocol as described by Bancroft and Gamble (2002). First, the ALA and non-ALA tissues were deparaffinized with two changes of xylene for 5 min each, followed by rehydration with two changes of absolute, 70% and 50% alcohols for 3 min each and washing in running tap water for 5 min. Tissues were then blocked with 3% hydrogen peroxide for 5 min, dipped in distilled water for 5 min and followed by 30 min incubation with 1:100 dilution of the corresponding polyclonal hamster serum sample i.e. sera from the ALA-induced hamster and control hamster used for the infected and control tissues, respectively. Washing steps were then carried out five times with PBSTween 20 (PBST), 2 min each. Tissues were incubated with 1:1 000 dilution of HRP-conjugated anti-hamster antibody (Sigma-Aldrich, USA) for 30 min and again washed with PBST. After washing, the tissues were developed with 3,3'-diaminobenzidine (DAB) substrate solution for 3 min and again washed with PBST. Finally, the tissues were counterstained with Harris's haematoxylin (Sigma-Aldrich, USA) for 1 min, followed by washing, differentiation with 1% acid alcohol, bluing with ammonia water, another washing step, dehydration with increasing graded alcohols, clearance with xylene and then mounted with DPX.

3. RESULTS AND DISCUSSION

The procedure involved in maintaining the HM1:IMSS axenic strain of *E. histolytica* trophozoites is not as straight-forward as culturing cancer cell lines, where chemically defined media such as RPMI and DMEM are commercially available. Here, the TYI-S-3 medium has to be freshly prepared and precautions have to be taken to ensure that each batch/lot of chemicals such as biosate peptone, vitamin solution and bovine serum are compatible and non-toxic to the amoebas. In addition, during the autoclaving process, the medium must be allowed to cool at room temperature when the autoclave temperature reaches 90 °C. This is to prevent the caramelisation or browning of sugar during the long duration in the autoclave, which is detrimental to the growth of the amoebas.

Since March 2009, the *E. histolytica* HM1:IMSS axenic strain has been successfully maintained in the Biomedicine Laboratory of School of Health Sciences. This has generated numerous applied and basic research projects involving *E. histolytica*. In trying to preserve the amoebas, the longest duration in which the amoebas could be revived from -80 °C was only about one week. Similar difficulty was reported by Clark (http://entamoeba.lshtm.ac.uk/cryo.htm), who cryopreserved the amoebas in liquid nitrogen. He concluded that the axenic amoeba cells are much more sensitive than many other cells.

In the animal experimentation, it was pertinent to use hamster as it is susceptible to *E. histolytica* infection. Experimentally induced ALA hamster normally dies after 7-10 days post-inoculation of the amoebas via the portal vein. Thus, cardiac puncture was performed on the morbid animal to obtain serum sample from hamster with confirmed

ALA, which is based on our success in culturing the virulent *E. histolytica* strain obtained from infected liver tissues. As expected the ~7-day post-infection antibody titre was low but by optimising the primary antibody dilution at 1:50, Western blot analysis revealed some interesting results. Animal experimentation is important as obtaining truly human ALA serum samples is very difficult as current diagnosis of ALA in patients depends mainly on ultrasonic and serological investigations. Confirmation of ALA based on identification of *E. histolytica* trophozoites in abscessed liver biopsy is considered invasive and unethical as most trophozoites are found in the healthy liver cells at the peripheral of the liver abscess.

In the Western blot analysis on 31 hamster ALA serum samples, the ~77 kDa protein was found to be a potential diagnostic biomarker (Figure 1). The protein was recognized by 26/31 (83.87%) ALA serum samples, but not detected by any of the healthy hamster sera. MALDI-TOF mass spectrometry revealed that the protein has pI value of 5.91, and the amino acid sequence was similar to the acetyl-CoA syntase (NCBI C4LUV9) in *Entamoeba histolytica* database.

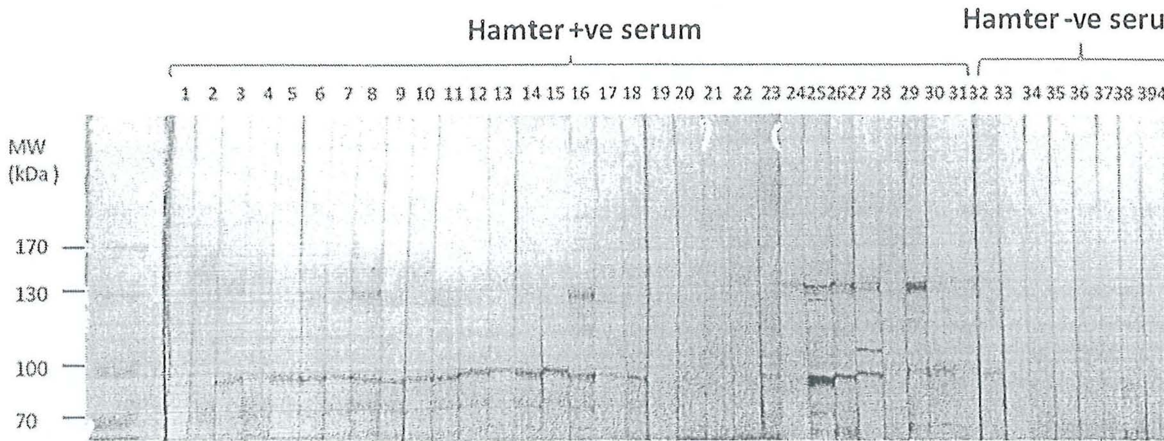


Figure 1. Representative Western/IgG blot of *E. histolytica* crude soluble antigen when probed with hamster serum samples. Lane 1-31: Individual hamster ALA serum samples (n=31); Lane 32: Hamster ALA pooled sera; Lane 33-38: Healthy individual hamster serum samples (n=6); Lane 39: Healthy hamster pooled serum samples; Lane 40: TBS control.

Besides delivering the two main objectives of this project, further experiments were performed in relation to diagnosis of amoebiasis. We also introduced the Eosin-Y stain as a rapid staining method for *E. histolytica* trophozoites in spiked stool sample. Based on the technique, microscopy detection of the trophozoites can be observed almost instantaneously as compared to the 2-hour period required by the routine Wheatley Trichrome staining technique. The compound phloxine-B in Eosin-Y stain was reportedly used to stain nuclear structures in histological sections (SPI.Supplies, 2009). Until now, there were no reports on the use of phloxine-B to stain nucleus of *E. histolytica*. Here, we showed that Eosin-Y was just as accurate as Wheatley trichrome staining method in identification of trophozoites in stool samples. Besides staining the characteristic nuclear features of the trophozoites and/or the engulfed erythrocytes, it could be performed easily to give spontaneous results. Another major advantage of Eosin-Y staining technique is that the stained trophozoites could easily be visualized under 400X magnification (Figure 2).

Another additional finding was the application of hamster polyclonal antibody in immunohistochemical staining of *E. histolytica* trophozoites in hamster liver. This approach gave better visualization than either the routine haematoxylin & eosin staining or the periodic acid Schiff staining (Figure 3). This is probably because immunohistochemical staining is presumed to be more specific as it is the consequence of specific reactions of amoebic trophozoite and antibodies against them.

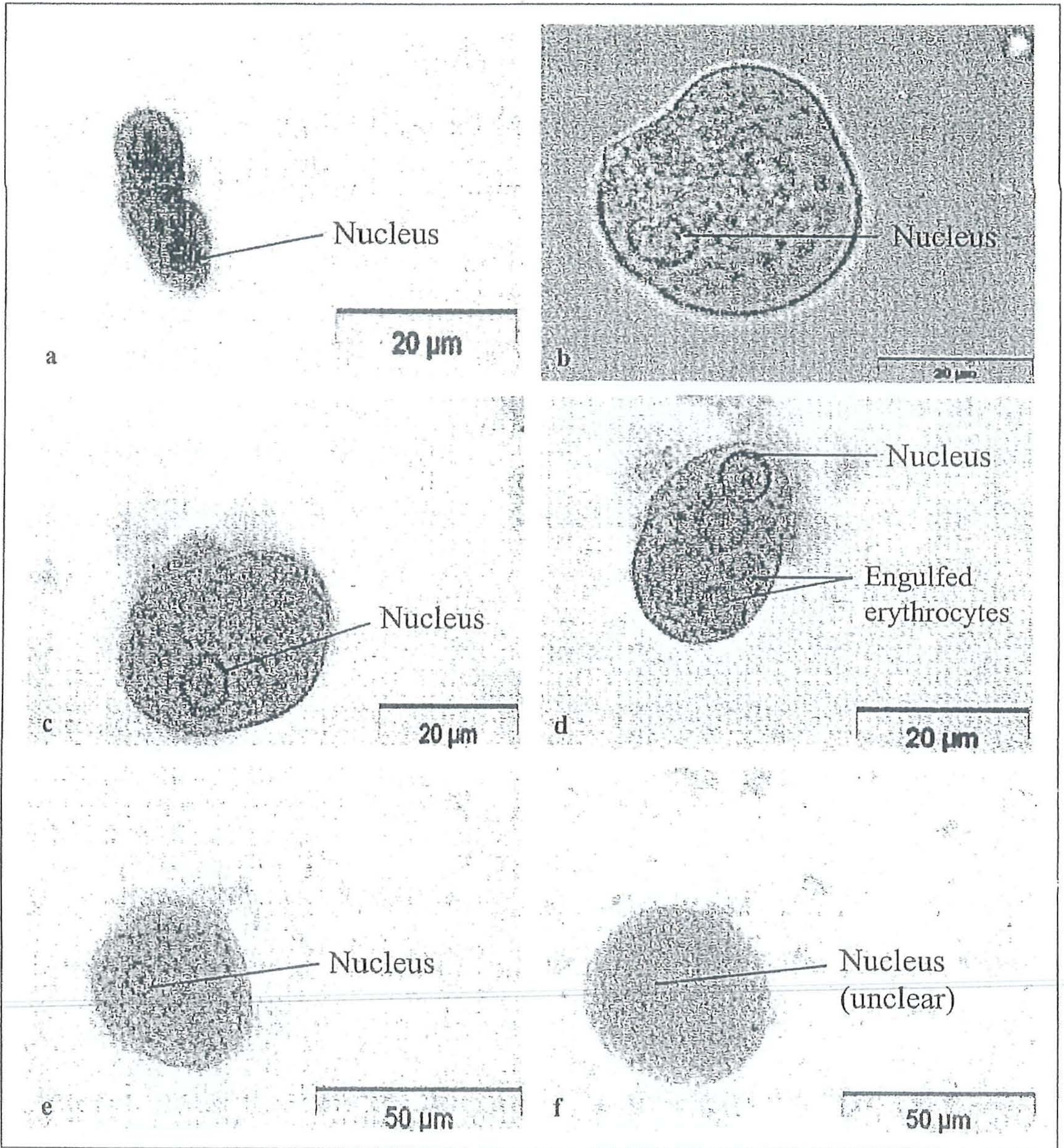


Figure 2. Stained trophozoites. (a) Wheatley trichrome stained trophozoite, 1000X magnification (b) Iodine stained trophozoite, 1000X magnification (c) Eosin-Y stained trophozoite, 1000X magnification (d) Eosin-Y stained erythrophagocytic trophoizoite, 1000X magnification (e) Eosin-Y stained trophozoite showed clear chromatin granules and karyosome, 400X magnification (f) Eosin-Y stained trophoizoite without DPX seal indicated unclear characteristics after an hour, 400 magnification

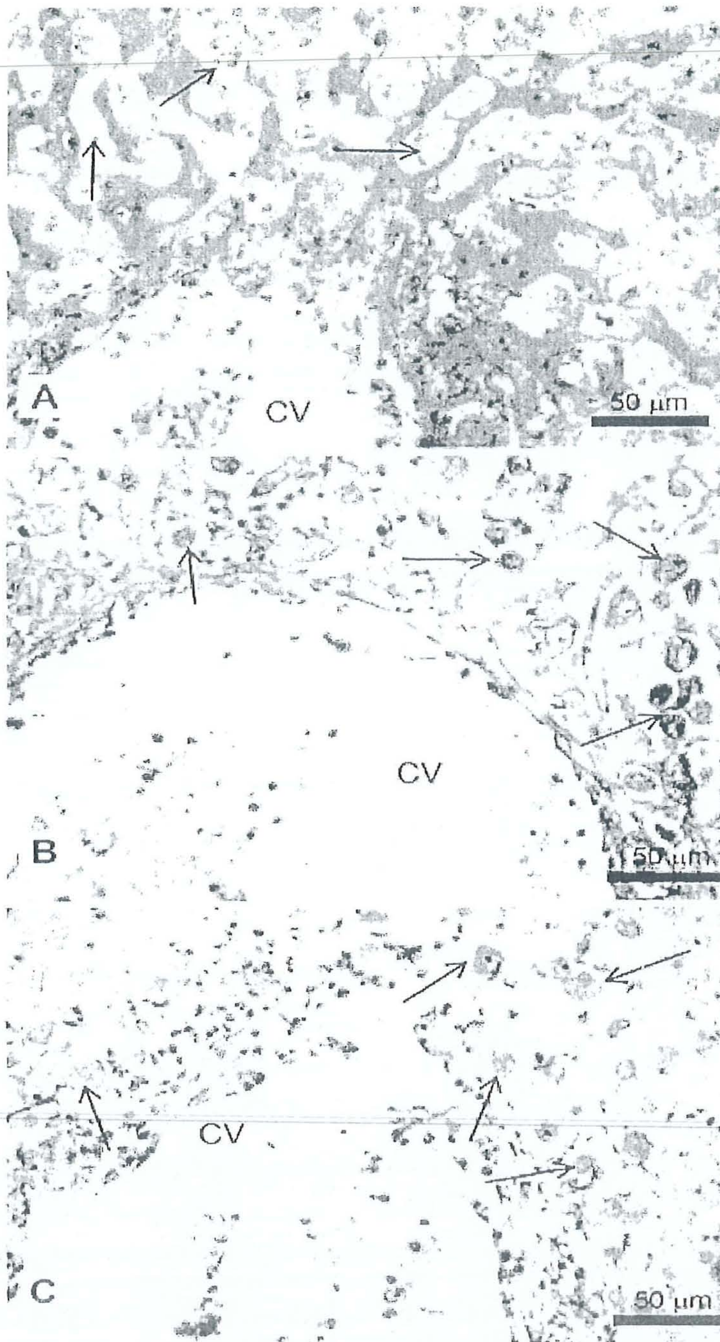


Figure 3. Micrographs indicating the different staining of E. histolytica trophozoites. A: H&E stain; B: PAS stain; C: IHC stain (400X); CV: Central vein. *E. histolytica* trophozoites are indicated with arrows. (A) Trophozoites (arrow) are visible as round, oval to pear shaped cells lying in lacunar spaces with occasional ingested red blood cells inside, very similar to macrophages in morphology. (B) PAS stained section showing the trophozoites (arrow) with magenta coloured cell membrane in a necrotic background. (C) IHC stain showing brown coloured trophozoites (arrow) with a distinct cell membrane easily identifiable against a background of necrosis and inflammation.

4. CONCLUSIONS

In conclusion, this research project has established the HM1:IMSS *E. histolytica* axenic strain of *E. histolytica* in Biomedicine Laboratory at the School of Health Sciences, USM. The availability of the amoeba culture has supported numerous applied and basic researches related to *E. histolytica* and will continue to facilitate future researches in better understanding the control of this simple yet deadly eukaryotic parasite. Among them is the rapid staining method for *E. histolytica* trophozoites in spiked stool sample using Eosin-Y stain, which allowed instantaneously observation of the parasites as compared to the 2-hour period required by the routine Wheatley Trichrome staining technique.

Another additional finding is the application of specific hamster polyclonal antibody in immunohistochemical staining of *E. histolytica* trophozoites in hamster liver. This approach gave better visualization than either the routine haematoxylin & eosin stain or the periodic acid Schiff stain. Thus, it is potentially important for diagnosis of ALA in identifying trophozoites in biopsied human liver samples.

The identification of Acetyl-CoA syntase (NCBI C4LUV9) via Western blot analysis using hamster ALA serum samples as a potential diagnostic biomarker for human amoebiasis shall be further explored in future studies.

ACKNOWLEDGEMENT

The authors would like to acknowledge Universiti Sains Malaysia for the research funding via the Research University Grant number 1001/PPSK/813009. We also wish to express our gratitude to the other contributing researchers for their input, namely Tan Zi Ning (USM Fellow), Wong Weng Kin (USM Fellow), Chan Chiat Han (USM Fellow), Khairul Nissa Saidin, Siti Shafiqah Anaqi Azham, Tan Chong Leong, Abdullah Bujang, Nik Zairi Zakaria,.Dr. Shyamoli Mustafa, Dr. Arefuddin Ahmed and Dr. See Too Wei Cun.

REFERENCES

Bancroft, J.D., Gamble, M. (2002). Theory and practice of histological techniques. 5th ed. China: Churchill Livingstone.

Clark, C.G. (2004). *Entamoeba histolytica* and *Entamoeba dispar*, the non-identical twins. In *The Pathogenic Enteric Protozoa: Giardia, Entamoeba, Cryptosporidium and Cyclospora* (eds. Sterling, C.R. & Adam, R.D.), pp. 15-26. MA: Kluwer Academic Publishers.

Diamond, L.S., Harlow, D.R. and Cunnick, C.C. (1978). A new medium for the axenic cultivation of *Entamoeba histolytica* and other *Entamoeba. Transaction of the Royal Society of Tropical Medicine and Hygiene* 72:431-432.

Goh, K.L., Wong, N.W., Paramsothy, M., Nojeg, M. & Somasundaram, K. (1987). Liver abscess in the tropics: experience in the University Hospital, Kuala Lumpur. *Postgraduate Medical Journal.* 63(741):551-554.

Jamaiah, I. & Shekhar, K.C. (1999). Amebiasis: A 10-year retrospective study at the University Hospital, Kuala Lumpur. *Medical Journal of Malaysia.* **54**(3): 296-302.

Juimo, A.G., Gervez, F. & Angwafo, F.F. (1992). Extraintestianl amebiasis. *Radiology.* 23: 249-250.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the 269 head of bacteriophage T4. *Nature* 227:680-685.

Mayer (2009). H & E staining Method and Protocol [Online] [Accessed 14/10/2009 9:19 AM]. Available from World Wide Web: http://www.ihcworld.com/protocols/ special stains/HE_Mayer.html

Olivos-Garcia A, Nequiz-Avendano M, Tello E, Martinez RD, Gonzalez-Canto A, Lopez-Vancell R, et al (2004). Inflammation, complement, ischemia and amoebic survival

in acute experimental amoebic liver abscesses in hamsters. *Experimental Molecular Pathology* *77*(1): 66-71.

Rajeswary, B. Sinniah, B. & Hasnah, H. (1994). Socio-economic factors associated with intestinal parasites among children living in Gombak. *Asia-Pacific Journal of Public Health* 7(1): 21-25.

Samarawickrema NA, Upcroft JA, Thammapalerd N, Upcroft P. (2001). A rapid-cooling method for cryopreserving Entamoeba histolytica. *Annals of Tropical Medicine and Parasitology* 95(8):853-855.

Stanley, S.L. (2003). Amoebiasis. *Lancet*. 361: 1025-1034.

Tanyuksel, M. & Petri, W.A. (2003). Laboratory diagnosis of amebiasis. *Clinical Microbiology Reviews*. 16(4): 713-729.

Wan Nor Azilah, W.A.W. (2007). Detection of *Entomoeba histolytica*-derived lectin using TECHLAB *Entamoeba histlytica* II ELISA in sera of patients with suspected ALA in HUSM. Master of Pathology (Microbiology) Thesis, U.S.M.

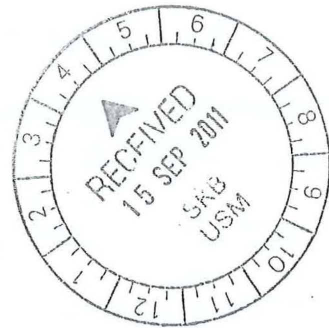
Weber C, Blazquez S, Marion S, Ausseur C, Vats D, Krzeminski M, et al. (2008) Bioinformatics and functional analysis of an Entamoeba histolytica mannosyltransferase necessary for parasite complement resistance and hepatical infection. *PLoS Neglected Tropical Diseases* 2(2): e165.

Wells, C.D. & Arguedas, M. (2004). Amebic liver abscess. *Southern Medical Journal*. 97: 673-682.

World Health Organisation (1995). The world health report: Bridging the gaps.

ANALYSIS OF *Entamoeba histolytica* ANTIGENS FOR IMPROVED DIAGNOSIS OF AMOEBIASIS

By



LIM BOON HUAT[1]
RAHMAH NOORDIN[2]
LALITHA PATTABHIRAMAN[3]
ALFONSO OLIVOS GARCIA[4]
ZEEHAIDA MOHAMED[5]
RUMAIZI SHAARI[6]

[1]School of Health Sciences, [2]USM Health Campus INFORMM, USM Main Campus; [3]Asian Institute of Medicine, Science and Technology, Kedah; [4]Department of Experimental Medicine, Universidad Nacional Autonoma de Mexico; [5]School of Medical Sciences, USM Health Campus; [6]School of Dental Sciences, USM Health Campus

PROJECT FUNDED BY UNIVERSITI SAINS MALAYSIA RESEARCH UNIVERSITY GRANT [1001/PPSK/813009]
10th October 2007 – 31st March 2011

ABSTRACT

Amoebiasis is one of the three most common fatal parasitic diseases in developing countries. Fatality is primarily due to amoebic liver abscess (ALA), an important cause of space-occupying lesions of liver due to invasion of *Entamoeba histolytica* via the colonic mucosa. This research project was undertaken to establish and maintain an axenic culture of *E. histolytica* in Universiti Sains Malaysia; then utilized it to identify and subsequently characterize a specific *E. histolytica* antigen that has diagnostic value via animal experimentation.

The HM1:IMSS *E. histolytica* axenic strain was established and maintained since March 2009 in Biomedicine Laboratory at School of Health Sciences, Universiti Sains Malaysia, in the customized TY1-S-33 medium. Preservation of the amoeba at -80 °C was successful up to approximately 7 days. As such, the culture is still continuously maintained at 36 °C to sustain the *E. histolytica* project. In the animal study, ALA was experimentally developed in Syrian golden hamster by inoculating *E. histolytica* trophozoites into the portal vein. Cardiac puncture was later performed on the morbid hamsters to obtain the ALA serum samples. Based on Western blot analyses on the ALA sera, the ~77 kDa protein was identified as a potential diagnostic biomarker, as it was recognized by 26/31 (83.87%) hamster ALA serum samples, but not detected by any of the healthy hamster sera. MALDI-TOF mass spectrometry results revealed that the protein has a pI value of 5.91 and the amino acid sequence was similar to acetyl-CoA syntase (NCBI C4LUV9) in *Entamoeba histolytica* database.

In addition to delivering the two objectives, other studies related to diagnosis of amoebiasis were performed. For instance, we also reported a rapid staining method to

detect *E. histolytica* trophozoites in spiked stool sample using Eosin-Y stain. This microscopy staining technique enabled almost instantaneously detection of the trophozoites as compared to the 2-hour preparation period required by the routine Wheatley Trichrome staining technique. Another additional contribution is the application of specific hamster polyclonal antibody in immunohistochemical staining of *E. histolytica* trophozoites in hamster liver. This approach gave better visualization of the trophozoites than either the routine haematoxylin & eosin stain or the periodic acid Schiff stain.

KEYWORDS: *Entamoeba histolytica*, amoebiasis, amoebic liver abscess (ALA), hamster, Eosin-Y stain, immunohistochemical (IHC) stain, Western blot and acetyl-CoA syntase

*Corresponding author: Tel: +6097619; Fax: +6097677515; limbh493@gmail.com

1. INTRODUCTION

Amoebiasis is a parasitic infection acquired through ingestion of food or water contaminated with cysts of *E. histolytica*. The parasite causes intestinal infection, diarrhoea and extraintestinal amoebiasis. The estimated global burden of the disease in humans is about 50 million with up to 100,000 deaths annually (Tanyuksel & Petri, 2003; Wells & Arguedas, 2004). In Malaysia, 9.9 % of children were infected (Rajeswary et al., 1994) and about 40% of liver abscess cases were found to be amoebic in origin (Goh et al., 1987; Jamaiah & Shekhar, 1999).

Despite its inherent drawbacks, microscopy continues to be the diagnostic method used in most laboratories around the world (Clark, 2004). The existing Wheatley trichrome staining technique is time-consuming as it requires at least 2-hour to prepare, and the stain cannot differentiate *E. histolytica* from non-pathogenic *E. dispar* (Tanyuksel & Petri, 2003). Unfortunately, only ~12% of patients with amoebic liver abscess (ALA) were reported with the amoebas in their stool samples (Wells & Arguedas, 2004). *E. histolytica* trophozoites were reported in up to 85% of liver abscess (Juimo et al., 1992), but aspiration of the abscess material is both invasive and technically demanding (Stanley, 2003). Culture/zymodeme analysis is specific; however one species may outgrow the other in cultures of specimens from mixed infections (Wells & Arguedas, 2004). Molecular techniques are highly sensitive and specific but they require a longer time to perform, technically complex, and also relatively costly (Stanley, 2003; Tanyuksel & Petri, 2003). A promising commercially available noninvasive antigen detection test was reported to detect circulating galactose/N-Acetyl galactosamine-specific lectin (or Gal/GalNac lectin) antigen in sera of 96% (22/23) ALA patients (Jamaiah & Shekhar, 1999). However, in another

study, the test kit detected the antigen in only 2.3% (1/43) of patients suspected of having ALA; although 76.7% (33/43) of them were found to be positive for anti-lectin antibody (Wan Nor Azilah, 2007).

Hence this study aimed to establish an axenic culture of *E. histolytica* in Biomedicine Laboratory of School of Health Sciences, Universiti Sains Malaysia, and subsequently utilizes the amoeba for research to improve on the existing diagnostic tools. Obtaining truly human ALA serum samples for research is difficult, as current diagnosis depends on ultrasonic and serological investigations. Technically, confirmation of ALA should be based on identification of *E. histolytica* trophozoites in abscessed liver biopsy, but this approach is invasive and considered unethical as most trophozoites are found in the healthy liver cells at the peripheral of the liver abscess. Thus, this study also aimed to identify and characterize a specific *E. histolytica* antigen that has diagnostic value via animal experimentation, in which ALA will be developed in susceptible hamster to obtain truly ALA serum samples for Western blot analysis.

2.1 Maintenance of *E. histolytica* trophozoites

2.1(a) Preparation of TYI-S-33 medium for *E. histolytica* culture

To prepare 1 L of TYI-S-33 medium, solutions A and B have to be prior prepared. Solution A was prepared by dissolving 30 g of Biosate peptone (Becton Dickinson, Lot No: 7050570) and 10 g Dextrose in 500 mL of distilled water. For solution B, 1 g of Dipotassium phosphate (K_2HPO_4), 0.6 g of Monopotassium phosphate (KH_2PO_4), 2 g of Sodium chloride (NaCl), 1g of Cysteine and 0.2 g of ascorbic acid were dissolved in 370 mL of distilled water. Solutions A and B were then mixed and subsequently added with 22.5 mg of ferric ammonium citrate. The pH of the solution was adjusted to 6.8 with 2 N NaOH, and filtered (Whatman, Cat. No: 1004240) before it was autoclaved at 121 °C for 10 minutes. When the autoclave temperature dropped to 90 °C, the bottle of solution was taken out and allowed to cool until it reached room temperature. Then, 30 mL of vitamin solution (SAFC Bioscience, Lot No: 7L0326) and 100 mL of decomplemented bovine serum (Invitrogen, Lot No: 667195) were added into the sterilized solution to form the TYI-S-33 medium, which was kept at 4 °C until it was ready to be used.

2.1(b) Preparation of Streptomycin-Benzylpenicillin-Kananmycin Antibiotics

Six gram of streptomycin sulfate and 3.6 g of benzylpenicillin sodium salt were dissolved in 6 mL of distilled water. Then, 2 g of kanamycin was added. The mixture was syringe-filtered through 0.20 µm filter disc (Schleicher & Schuell, Lot No: DR0700-3) and kept in 4 °C.

2.1(c) Viability Testing

First, a 1:1 dilution of amoeba suspension with 0.4% Trypan blue solution was prepared. Next, the mixture was loaded onto a hemocytometer counting chamber. The number of unstained amoebas (viable cells) and stained (non-viable cells) were counted. Finally, the viability of amoeba was determined based on the calculations below:

Total number of viable cells = average number of viable cells x 10^4 x dilution factor

= n x 10^4 x 2

$$\text{Viability of amoeba} = \frac{\text{Total number of viable cells} \times 100\ \%}{\text{Total number of cells}}$$

2.1(d) Hemocytometer Counting

A clean cover-slip was placed on a clean hemocytometer counting chamber. A drop of amoeba suspension was loaded at the edge of the chamber and the suspension was allowed to be drawn into the chamber by capillary action. The chamber was then placed on the microscope stage and the average number of amoeba was determined using the formula below.

Formula: c= n/v

Where c = cell suspension in cells/mL; n = average number of cells/mm^2 area and v = volume counted = 10^4 (1mm^2 = 0.1 mm^3 = 10^{-4}mL).

Thus, c = n x 10^4

2.1(e) Harvesting of Amoebic Culture

Prior to culturing of the axenic *E. histolytica*, a viability test was performed. Then, an appropriate amount of the amoeba was transferred into each culture tube containing 7.5 mL of TYI-S-3 medium. The culture tube was placed in a horizontal position 36 °C for 72 hr. (Diamond et al., 1978). The amoebas were harvested by first cooling the culture tubes on ice for 5 minutes. Then, the culture medium were transferred into sterile 1.5 mL microcentrifuge tubes and spun at 440 x g for 3 minutes at 4 °C. After decanting the medium, 1 mL of sterile 1X PBS was added to resuspend the amoeba pellet. The amoeba suspension was again washed by centrifugation. After removing the supernatant, the amoeba pellet was stored at -80 °C.

2.1(f) Maintenance of Virulent *E. histolytica* and Collection of Hamster ALA Serum Samples

To maintain the virulence of axenic *E. histolytica* (HM1-IMSS), each fortnight the amoebas in TYI-S-3 medium were used to develop ALA in a Syrian golden hamster (*Mesocricetus auratus*). The experimental development of ALA in hamster was performed as described by Olivos-Garcia et al. (2004) and Weber et al. (2008). Briefly, 1 x 10^6 trophozoites suspended in 0.2 mL phosphate buffer saline (PBS) was inoculated into the portal vein of an anesthetized (6 mg pentobarbital / 100 g body weight) male hamster. To prevent excessive bleeding, a small piece of gelfoam (Pharmacia & Upjohn Co., USA) was placed onto the injected site of portal vein and held for one minute with a piece of gauze. About a week later, the morbid animal was sacrificed with 3X overdose of pentobarbital. Cardiac puncture was performed on the euthanized animal to collect the hamster ALA serum sample. Following which, the abscessed liver was transferred aseptically onto a sterile Petri dish and cut into small fragments. Some fragments were transferred into a sterile culture tube and filled with 7.5 mL of TYI-S-33 medium followed

by adding 15 μL of streptomycin-benzylpenicillin-kananmycin antibiotics. The culture tube was then incubated at 36°C in a horizontal position. After 24 hours of incubation, the tube wall was observed under inverted microscope to check for amoebas. The medium was replaced with fresh medium and antibiotics. The procedure was repeated each 24 hours for 5 days, in which the antibiotics were gradually reduced. Finally, the amoebas were cultured in the absence of antibiotics and ready to be harvest in TYI-S-33 medium. The animal experimentation above was approved by USM Animal Research Ethics Committee [No. Animal Ethics Approval: USM/Animal Ethics Approval/ 2008/(40)(129)].

2.1(g) Preservation of *E. histolytica*

Preservation of *E. histolytica* was performed as suggested by Samarawickrema et al. (2001) with modifications. Following the DMSO stop, the amoebas was immediately preserved in a -80 °C freezer instead of being first subjected to rapid cooling at -70 °C for 48 hr, followed by cryopreservation in liquid nitrogen.

2.2 Analysis of *E. histolytica* antigens

2.2 (a) SDS-PAGE

Protein samples were electrophoretically separated via SDS-PAGE using Bio-Rad Mini Protean III Electrophoresis Cell and Protean® II xi Cell according to Laemmli (1970) protocol with modifications. Prior to SDS-PAGE, crudes soluble antigen of *E. histolytica* was mixed with 2X Laemmli sample buffer and boiled for 5 min. Subsequently, it was separated using 6% or 10% SDS-PAGE gel, at constant current of 25 mA per gel for about 1 h.

2.2 (b) Western blotting

Upon completion of SDS-PAGE, proteins in the gel was electrophoretically transferred onto a 0.45 µm nitrocellulose membrane (NCP) using semi-dry transblot (Bio Rad, USA) at a constant voltage of 15 V for 30 min. The NCP was blocked for 1hr at RT with 5 % skim milk prepared in 10 mM Tris buffered saline, pH 7.2 (TBS). Subsequently, the NCP was washed (3 x 5 min) with TBS containing 0.1 % Tween-20 (TBS-T). Then, the NCP was cut into multiple strips and incubated with hamster sera at dilution of 1:50 (in TBS-T) for 2 hours at RT. The NCP strips were then washed three times with TBS-T, and then incubated with monoclonal mouse anti–hamster IgG conjugated with horseradish peroxides (HRP) at dilution of 1:4000 for 1hr. Subsequently, the NCP strips were again washed (3 x 5 min) with TBS-T. Western blot substrates *i.e.* enhanced chemiluminescence (ECL) blotting reagent (Roche diagnostics, Germany) or tetramethylbenzidine (TMB) substrate for membrane (Sigma, USA) were used as substrates. The Western blot signal was captured using camera (Lumix, Germany).

2. 3 Rapid Eosin-Y Staining

The Eosin-Y stain (Sigma HT110316, USA) used in this study was in its working dilution, thus was applied directly on stool samples without fuss. Alternatively, Eosin-Y staining solution can be prepared by mixing 1% (w/v) Eosin-Y, 1% (w/v) phloxine-B, 95% ethanol and glacial acetic acid in appropriate volumes (Mayer, 2009). Briefly, approximately one million axenic *E. histolytica* was washed with 1X PBS and spiked in 2 g of fresh semi-solid stool sample obtained from a healthy volunteer. An applicator stick was used to mix ~30 µL of the Eosin-Y stain with ~2 mg of stool sample on a clean slide. DPX was used to seal the cover slip placed over the sample to the slide, which was then observed under a microscope at 400X and 1000X magnification. The images of the stained

trophozoites were captured using Olympus Image Analysis System (Olympus System Microscope Model BX41, Japan).

2.4. Immunohistochemical Staining

Indirect staining was performed on processed tissue sections with some modifications on the standard protocol as described by Bancroft and Gamble (2002). First, the ALA and non-ALA tissues were deparaffinized with two changes of xylene for 5 min each, followed by rehydration with two changes of absolute, 70% and 50% alcohols for 3 min each and washing in running tap water for 5 min. Tissues were then blocked with 3% hydrogen peroxide for 5 min, dipped in distilled water for 5 min and followed by 30 min incubation with 1:100 dilution of the corresponding polyclonal hamster serum sample i.e. sera from the ALA-induced hamster and control hamster used for the infected and control tissues, respectively. Washing steps were then carried out five times with PBSTween 20 (PBST), 2 min each. Tissues were incubated with 1:1 000 dilution of HRP-conjugated anti-hamster antibody (Sigma-Aldrich, USA) for 30 min and again washed with PBST. After washing, the tissues were developed with 3,3'-diaminobenzidine (DAB) substrate solution for 3 min and again washed with PBST. Finally, the tissues were counterstained with Harris's haematoxylin (Sigma-Aldrich, USA) for 1 min, followed by washing, differentiation with 1% acid alcohol, bluing with ammonia water, another washing step, dehydration with increasing graded alcohols, clearance with xylene and then mounted with DPX.

3. RESULTS AND DISCUSSION

The procedure involved in maintaining the HM1:IMSS axenic strain of *E. histolytica* trophozoites is not as straight-forward as culturing cancer cell lines, where chemically defined media such as RPMI and DMEM are commercially available. Here, the TYI-S-3 medium has to be freshly prepared and precautions have to be taken to ensure that each batch/lot of chemicals such as biosate peptone, vitamin solution and bovine serum are compatible and non-toxic to the amoebas. In addition, during the autoclaving process, the medium must be allowed to cool at room temperature when the autoclave temperature reaches 90 °C. This is to prevent the caramelisation or browning of sugar during the long duration in the autoclave, which is detrimental to the growth of the amoebas.

Since March 2009, the *E. histolytica* HM1:IMSS axenic strain has been successfully maintained in the Biomedicine Laboratory of School of Health Sciences. This has generated numerous applied and basic research projects involving *E. histolytica*. In trying to preserve the amoebas, the longest duration in which the amoebas could be revived from -80 °C was only about one week. Similar difficulty was reported by Clark (http://entamoeba.lshtm.ac.uk/cryo.htm), who cryopreserved the amoebas in liquid nitrogen. He concluded that the axenic amoeba cells are much more sensitive than many other cells.

In the animal experimentation, it was pertinent to use hamster as it is susceptible to *E. histolytica* infection. Experimentally induced ALA hamster normally dies after 7-10 days post-inoculation of the amoebas via the portal vein. Thus, cardiac puncture was performed on the morbid animal to obtain serum sample from hamster with confirmed

ALA, which is based on our success in culturing the virulent *E. histolytica* strain obtained from infected liver tissues. As expected the ~7-day post-infection antibody titre was low but by optimising the primary antibody dilution at 1:50, Western blot analysis revealed some interesting results. Animal experimentation is important as obtaining truly human ALA serum samples is very difficult as current diagnosis of ALA in patients depends mainly on ultrasonic and serological investigations. Confirmation of ALA based on identification of *E. histolytica* trophozoites in abscessed liver biopsy is considered invasive and unethical as most trophozoites are found in the healthy liver cells at the peripheral of the liver abscess.

In the Western blot analysis on 31 hamster ALA serum samples, the ~77 kDa protein was found to be a potential diagnostic biomarker (Figure 1). The protein was recognized by 26/31 (83.87%) ALA serum samples, but not detected by any of the healthy hamster sera. MALDI-TOF mass spectrometry revealed that the protein has pI value of 5.91, and the amino acid sequence was similar to the acetyl-CoA syntase (NCBI C4LUV9) in *Entamoeba histolytica* database.

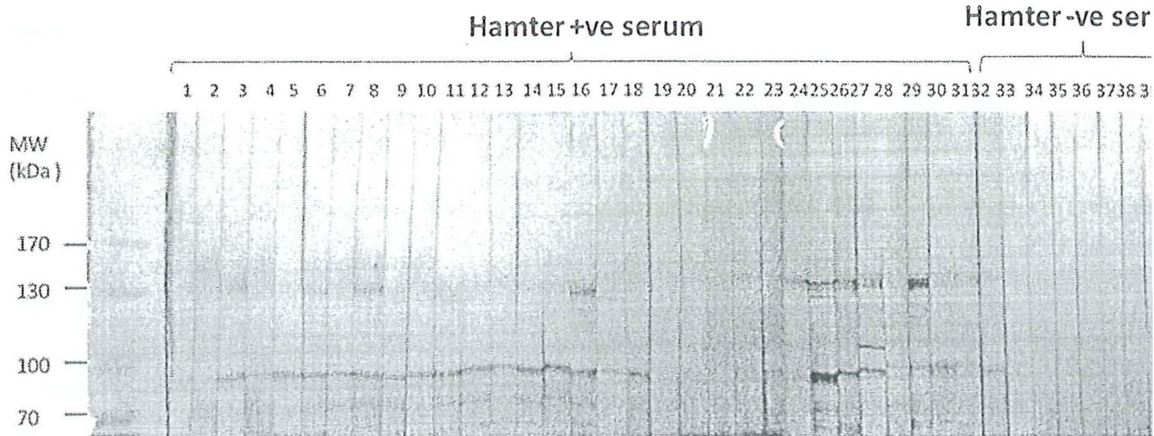


Figure 1. Representative Western/IgG blot of *E. histolytica* crude soluble antigen when probed with hamster serum samples. Lane 1-31: Individual hamster ALA serum samples (n=31); Lane 32: Hamster ALA pooled sera; Lane 33-38: Healthy individual hamster serum samples (n=6); Lane 39: Healthy hamster pooled serum samples; Lane 40: TBS control.

Besides delivering the two main objectives of this project, further experiments were performed in relation to diagnosis of amoebiasis. We also introduced the Eosin-Y stain as a rapid staining method for *E. histolytica* trophozoites in spiked stool sample. Based on the technique, microscopy detection of the trophozoites can be observed almost instantaneously as compared to the 2-hour period required by the routine Wheatley Trichrome staining technique. The compound phloxine-B in Eosin-Y stain was reportedly used to stain nuclear structures in histological sections (SPI.Supplies, 2009). Until now, there were no reports on the use of phloxine-B to stain nucleus of *E. histolytica*. Here, we showed that Eosin-Y was just as accurate as Wheatley trichrome staining method in identification of trophozoites in stool samples. Besides staining the characteristic nuclear features of the trophozoites and/or the engulfed erythrocytes, it could be performed easily to give spontaneous results. Another major advantage of Eosin-Y staining technique is that the stained trophozoites could easily be visualized under 400X magnification (Figure 2).

Another additional finding was the application of hamster polyclonal antibody in immunohistochemical staining of *E. histolytica* trophozoites in hamster liver. This approach gave better visualization than either the routine haematoxylin & eosin staining or the periodic acid Schiff staining (Figure 3). This is probably because immunohistochemical staining is presumed to be more specific as it is the consequence of specific reactions of amoebic trophozoite and antibodies against them.

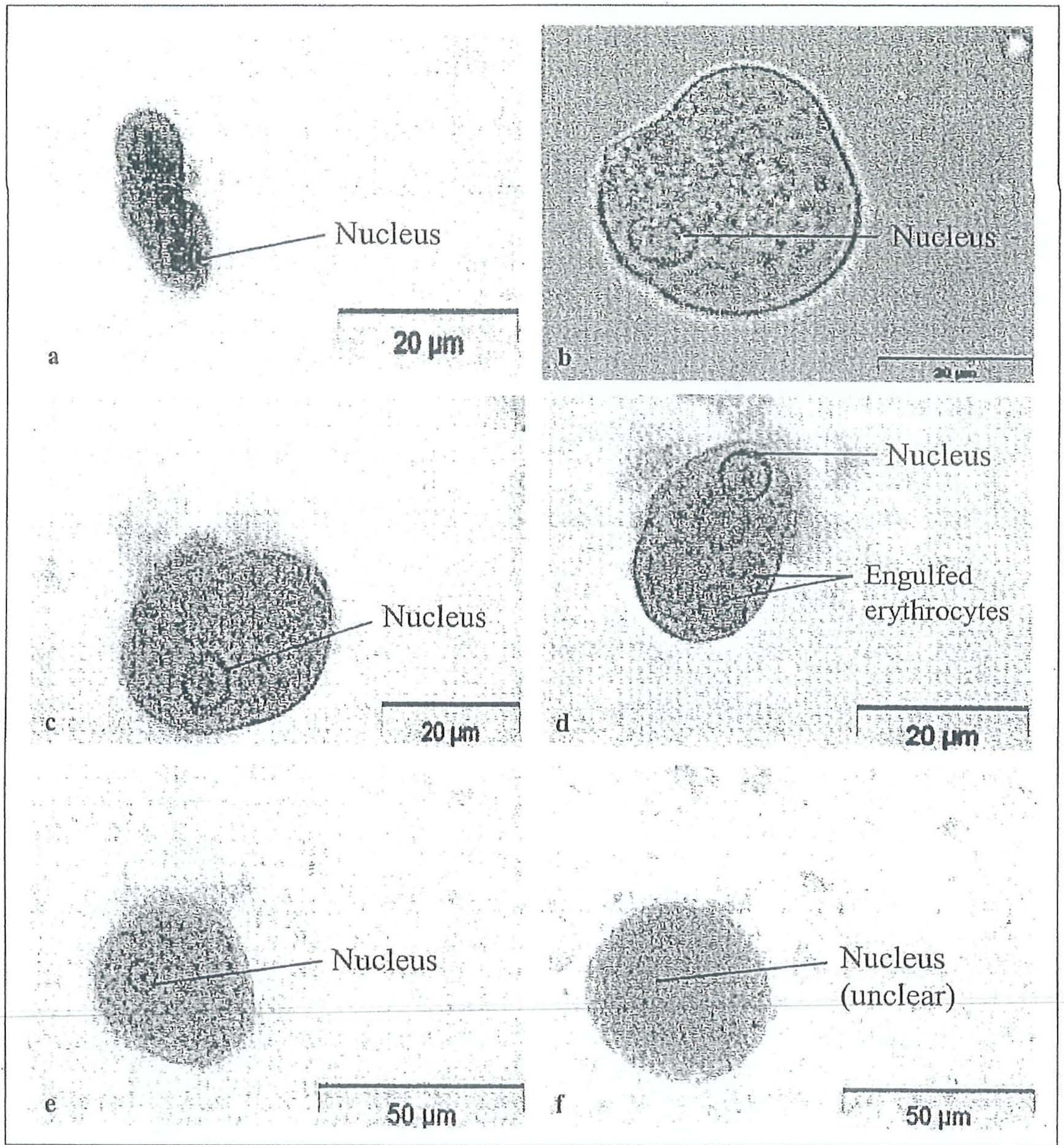


Figure 2. Stained trophozoites. (a) Wheatley trichrome stained trophozoite, 1000X magnification (b) Iodine stained trophozoite, 1000X magnification (c) Eosin-Y stained trophozoite, 1000X magnification (d) Eosin-Y stained erythrophagocytic trophoizoite, 1000X magnification (e) Eosin-Y stained trophozoite showed clear chromatin granules and karyosome, 400X magnification (f) Eosin-Y stained trophoizoite without DPX seal indicated unclear characteristics after an hour, 400 magnification

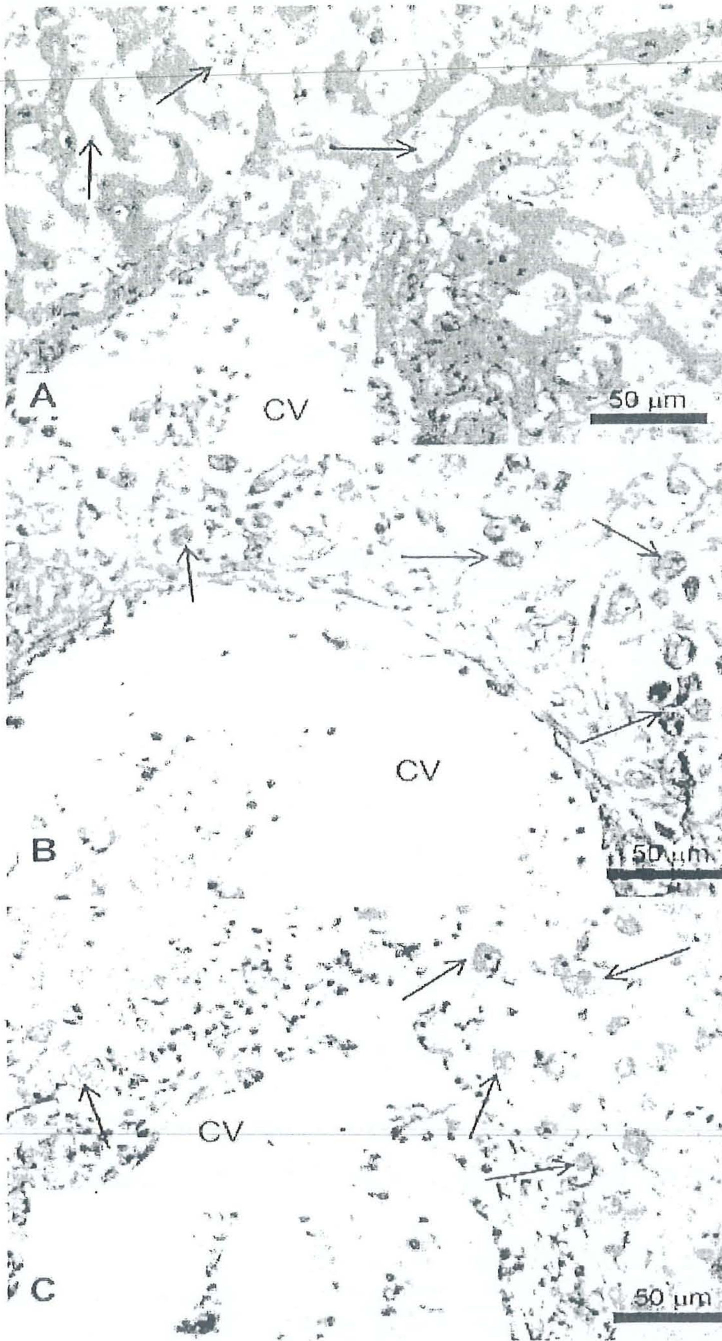


Figure 3. Micrographs indicating the different staining of E. histolytica trophozoites. A: H&E stain; B: PAS stain; C: IHC stain (400X); CV: Central vein. *E. histolytica* trophozoites are indicated with arrows. (A) Trophozoites (arrow) are visible as round, oval to pear shaped cells lying in lacunar spaces with occasional ingested red blood cells inside, very similar to macrophages in morphology. (B) PAS stained section showing the trophozoites (arrow) with magenta coloured cell membrane in a necrotic background. (C) IHC stain showing brown coloured trophozoites (arrow) with a distinct cell membrane easily identifiable against a background of necrosis and inflammation.

4. CONCLUSIONS

In conclusion, this research project has established the HM1:IMSS *E. histolytica* axenic strain of *E. histolytica* in Biomedicine Laboratory at the School of Health Sciences, USM. The availability of the amoeba culture has supported numerous applied and basic researches related to *E. histolytica* and will continue to facilitate future researches in better understanding the control of this simple yet deadly eukaryotic parasite. Among them is the rapid staining method for *E. histolytica* trophozoites in spiked stool sample using Eosin-Y stain, which allowed instantaneously observation of the parasites as compared to the 2-hour period required by the routine Wheatley Trichrome staining technique.

Another additional finding is the application of specific hamster polyclonal antibody in immunohistochemical staining of *E. histolytica* trophozoites in hamster liver. This approach gave better visualization than either the routine haematoxylin & eosin stain or the periodic acid Schiff stain. Thus, it is potentially important for diagnosis of ALA in identifying trophozoites in biopsied human liver samples.

The identification of Acetyl-CoA syntase (NCBI C4LUV9) via Western blot analysis using hamster ALA serum samples as a potential diagnostic biomarker for human amoebiasis shall be further explored in future studies.

ACKNOWLEDGEMENT

The authors would like to acknowledge Universiti Sains Malaysia for the research funding via the Research University Grant number 1001/PPSK/813009. We also wish to express our gratitude to the other contributing researchers for their input, namely Tan Zi Ning (USM Fellow), Wong Weng Kin (USM Fellow), Chan Chiat Han (USM Fellow), Khairul Nissa Saidin, Siti Shafiqah Anaqi Azham, Tan Chong Leong, Abdullah Bujang, Nik Zairi Zakaria,.Dr. Shyamoli Mustafa, Dr. Arefuddin Ahmed and Dr. See Too Wei Cun.

Bancroft, J.D., Gamble, M. (2002). Theory and practice of histological techniques. 5th ed. China: Churchill Livingstone.

Clark, C.G. (2004). *Entamoeba histolytica* and *Entamoeba dispar*, the non-identical twins. In *The Pathogenic Enteric Protozoa: Giardia, Entamoeba, Cryptosporidium and Cyclospora* (eds. Sterling, C.R. & Adam, R.D.), pp. 15-26. MA: Kluwer Academic Publishers.

Diamond, L.S., Harlow, D.R. and Cunnick, C.C. (1978). A new medium for the axenic cultivation of *Entamoeba histolytica* and other *Entamoeba. Transaction of the Royal Society of Tropical Medicine and Hygiene* 72:431-432.

Goh, K.L., Wong, N.W., Paramsothy, M., Nojeg, M. & Somasundaram, K. (1987). Liver abscess in the tropics: experience in the University Hospital, Kuala Lumpur. *Postgraduate Medical Journal.* 63(741):551-554.

Jamaiah, I. & Shekhar, K.C. (1999). Amebiasis: A 10-year retrospective study at the University Hospital, Kuala Lumpur. *Medical Journal of Malaysia.* **54**(3): 296-302.

Juimo, A.G., Gervez, F. & Angwafo, F.F. (1992). Extraintestianl amebiasis. *Radiology.* 23: 249-250.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the 269 head of bacteriophage T4. *Nature* 227:680-685.

Mayer (2009). H & E staining Method and Protocol [Online] [Accessed 14/10/2009 9:19 AM]. Available from World Wide Web: http://www.ihcworld.com/protocols/ special stains/HE_Mayer.html

Olivos-Garcia A, Nequiz-Avendano M, Tello E, Martinez RD, Gonzalez-Canto A, Lopez-Vancell R, et al (2004). Inflammation, complement, ischemia and amoebic survival

in acute experimental amoebic liver abscesses in hamsters. *Experimental Molecular Pathology* 77(1): 66-71.

Rajeswary, B. Sinniah, B. & Hasnah, H. (1994). Socio-economic factors associated with intestinal parasites among children living in Gombak. *Asia-Pacific Journal of Public Health* 7(1): 21-25.

Samarawickrema NA, Upcroft JA, Thammapalerd N, Upcroft P. (2001). A rapid-cooling method for cryopreserving Entamoeba histolytica. *Annals of Tropical Medicine and Parasitology* 95(8):853-855.

Stanley, S.L. (2003). Amoebiasis. *Lancet*. 361: 1025-1034.

Tanyuksel, M. & Petri, W.A. (2003). Laboratory diagnosis of amebiasis. *Clinical Microbiology Reviews*. 16(4): 713-729.

Wan Nor Azilah, W.A.W. (2007). Detection of *Entomoeba histolytica*-derived lectin using TECHLAB *Entamoeba histlytica* II ELISA in sera of patients with suspected ALA in HUSM. Master of Pathology (Microbiology) Thesis, U.S.M.

Weber C, Blazquez S, Marion S, Ausseur C, Vats D, Krzeminski M, et al. (2008) Bioinformatics and functional analysis of an Entamoeba histolytica mannosyltransferase necessary for parasite complement resistance and hepatical infection. *PLoS Neglected Tropical Diseases* 2(2): e165.

Wells, C.D. & Arguedas, M. (2004). Amebic liver abscess. *Southern Medical Journal*. 97: 673-682.

World Health Organisation (1995). The world health report: Bridging the gaps.

Tropical Biomedicine 26(3): 366–368 (2009)

Research Note

Seroprevalence of anti-amoebic antibody among blood donors by indirect hemeagglutination assay

Zeehaida, M.[1], Zairi, N.Z.[1], Tan, Z.N.[2], Wong, W.K.[2] and Lim, B.H.[2]
[1] Department of Medical Microbiology and Parasitology, School of Medical Sciences and
[2] School of Health Sciences, Universiti Sains Malaysia, Health Campus, 16150, Kubang Kerian, Kelantan
Email address: zeehaida@kck.usm.my
Received 21 September 2009; received in revised form 10 October 2009; accepted 12 October 2009

Abstract. The screening for anti-amoebic antibody among a group of donors was to obtain negative control serum samples for an on-going antigen development assay in diagnosis of amoebic liver abscess. Out of 200 samples, 125 (62.5%) were negative, whereas 44 (21.5%) had IHA titer of less than 1:128 and 31 (16.0%) of the samples had significant IHA titers of 1:128 or more, in which 2 serum samples gave titers of 1:4096.

The north-eastern state of peninsular Malaysia, Kelantan faces the South China Sea in the east and shares its border with Thailand in the north. It occupies an area of 15 020 sq. km and has a population of 1 478 800. Kelantan state is endemic for water-borne diseases, in which the overall incidence was less than 5 per 100 000 people from 2000 till 2004. The incidence of typhoid/paratyphoid in year 2004 was 1.87 per 100 000 population whereas the incidences of cholera, hepatitis A and dysentery were lower and accounted for 0.35, 0.42 and 0.43 per 100 000 population respectively (Ministry of Health Malaysia, 2004). Thus, according to the above figure, the estimated incidence of dysentery for this state is 6.36.

Though many pathogens cause dysentery, the prevalence of amoebiasis differs according to age, socioeconomic status and geographical distribution. There was no available data from previous publication on the background seropositivity of amoebiasis among healthy population in Malaysia. In Malaysia, blood donors are considered healthy adults aged more than 18 years, weighing more than 50 kg, clinically healthy and are seronegative for HIV, hepatitis B and C, syphilis and malaria. The screening for anti-amoebic antibody among this group of donors was to obtain negative control serum samples for an on-going antigen development assay in diagnosis of amoebic liver abscess. The serum samples were first screened by indirect hemagglutination assay (IHA), then followed by IHA titrations to select serum samples with titer of 1:64 or more, which were considered positive for amoebiasis, as suggested by the manufacturer (Dade-Behring Marburg, Germany).

Two hundred pooled serum samples from blood donors were screened by IHA; 125 (62.5%) were negative, whereas 44 (21.5%) and 31 (16.0%) had IHA titer of less than 1:128 and 1:128 or more titer respectively (Table 1). Detection of lower titers among blood donors could be due to previous exposure to amoebiasis among the local healthy population with either intestinal or extraintestinal forms. Thirty one

Table 1. Distribution of groups by IHA titers (n=200)

IHA titers	Number (percentage)
Negative	125 (62.5)
Less than 1:128	44 (21.5)
1:128 and more	31 (16.0)

Table 2. Distribution of IHA titers among the blood donors (n=200)

IHA titers	Number (percentage)
Negative	125 (62.5)
1:16	27 (13.5)
1:32	9 (4.5)
1:64	8 (4.0)
1:128	17 (8.5)
1:256	8 (4.5)
1:512	2 (1.0)
1:1024	2 (1.0)
1:2048	0 (0.0)
1:4096	2 (1:0)

(16.0%) of the samples had significant IHA titers of 1:128 or more, in which 2 serum samples gave titers of 1:4096 (Table 2). In these cases, the possibility of having some forms of amoebiasis at the time of blood donation could not be ruled out, neither the likelihood of being asymptomatic carriers.

In another report, the seroprevalence of villagers from West Kalimantan, Borneo who had IHA titers equal or greater than 1:128 was 7% (Cross *et al.*, 1976) as compared to this study which was 16.0%. The seroprevalence of blood donors from urban, suburban and rural population of Puebla State, Mexico which was done using IHA alone was 8.6% whereas when IHA and ELISA were employed together, as recommended by WHO, the seroprevalence was 6.4% (Sánchez-Guillén *et al.*, 2000).

Lower IHA titers had been demonstrated in patients with amoebic liver abscess (ALA) who were admitted to our hospital. In a previous study, 27.6% (16/58) ALA patients had antibody titer of 1:256 or less (Zeehaida *et al.*, 2008). The lower titers found in these patients could be due to low antibody levels in the early course of the disease.

The seroprevalence of amoebiasis among blood donors in this study was higher as compared to those reported previously in Malaysia and other surrounding endemic regions. The finding showed that the background seropositivity is significantly high among healthy population in this local setting. A lower titer of 1:128 could not be taken as a positive titer since it overlapped significantly with titers found among the blood donors. Thus, the titer had less value for diagnosis of extraintestinal amoebiasis. Supported by the clinical symptoms and signs of amoebiasis, 1:256 is deemed a significant titer for diagnosis of the disease, particularly in this local setting.

Acknowledgement. The study was partially funded by University Sains Malaysia Research University Grant, number: 1001/PPSK/813009

REFERENCES

Annual report, Ministry of Health Malaysia, 2004, page 88-89.

Cross, J.H., Clarke, M.D., Cole, W.C., Lien, J.C., Partono, F., Djakaria, Joesoef, A. & Oemijati, S. (1976). Parasitic infections in humans in West Kalimantan (Borneo), Indonesia. *Tropical Geography Medicine* 28(2): 121-30.

Sánchez-Guillén, M.C., Velázquez-Rojas, M., Salgado-Rosas, H., Torres-Rasgado, E., Pérez-Fuentes, R., Martínez-Munguía, J. & Talamás-Rohana, P. (2000). Seroprevalence of anti-*Entamoeba histolytica* antibodies by IHA and ELISA assays in blood donors from Puebla, Mexico. *Archives of Medical Research* **31** S53–S54.

Zeehaida, M., Wan Nor Amilah, W.A.W., Amry, A.R., Hassan, S., Sarimah, A. & Rahmah, N. (2008). A study on the usefulness of Techlab *Entamoeba histolytica* II antigen detection ELISA in the diagnosis of amoebic liver abscess (ALA) at Hospital Universiti Sains Malaysia (HUSM), Kelantan, Malaysia. *Tropical Biomedicine* **25**(3): 209–216.

Comparison of protein-free defined media, and effect of L-cysteine and ascorbic acid supplementation on viability of axenic *Entamoeba histolytica*

Weng Kin Wong · Zi Ning Tan · Boon Huat Lim · Zeehaida Mohamed · Alfonso Olivos-Garcia · Rahmah Noordin

Received: 29 June 2010 / Accepted: 8 September 2010 / Published online: 5 October 2010
© Springer-Verlag 2010

Abstract *Entamoeba histolytica* is the etiologic agent for amoebiasis. The excretory–secretory (ES) products of the trophozoites contain virulence factors and antigens useful for diagnostic applications. Contaminants from serum supplements and dead trophozoites impede analysis of ES. Therefore, a protein-free medium that can sustain maximum viability of *E. histolytica* trophozoites for the longest time duration will enable collection of contaminant-free and higher yield of ES products. In the present study, we compared the efficacy of four types of media in maintaining ≥95% trophozoite viability namely Roswell Memorial Park Institute (RPMI-1640), Dulbecco's Modified Eagle Med (DMEM), phosphate-buffered saline for amoeba (PBS and Hank's balanced salt solution (HBSS). Concurrently effect of adding L-cysteine and ascorbic acid (C&A) to medium on the parasite viability was also compared. DM and RPMI 1640 showed higher viabilities as compare PBS-A and HBSS. Only RPMI 1640 showed no statis difference with the control medium for the first 4 h, how the ≥95% viability was only maintained for the first 2 h. other protein-free media showed differences from the ser and vitamin-free TYI-S-33 control media even after 1 incubation. When supplemented with C&A, all media w found to sustain higher trophozoite viabilities than th without the supplements. HBSS-C&A, DMEM-C&A, RPMI 1640-C&A demonstrated no difference ($P>0.05$ parasite viabilities when compared with the control med throughout the 8-h incubation period. DMEM-C&A sho an eightfold increment in time duration of sustaining ≥9 parasite viability, i.e. 8 h, as compared to DMEM alo Both RPMI 1640-C&A and HBSS-C&A revealed four and threefold increments (i.e., 8 and 6 h, respective whereas PBS-A-C&A showed only onefold improvem (i.e., 2 h) as compared to the respective media without C& Thus, C&A-supplemented DMEM or RPMI are recommen for collection of ES products.

W. K. Wong
Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia

R. Noordin (✉)
Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia, 11800 Penang, Malaysia
e-mail: rahmah8485@gmail.com

Z. N. Tan · B. H. Lim
School of Health Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia

Z. Mohamed
Department of Medical Microbiology and Parasitology, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia

A. Olivos-Garcia
Departamento de Medicina Experimental, Facultad de Medicina, Universidad Nacional Autónoma de México, 04510 México, Mexico DF, Mexico

Introduction

Entamoeba histolytica is an enteric protozoan parasite t causes amoebiasis. This disease affects more than million people around the world and causes up to 100,0 fatal cases annually (Que and Reed 2000). This cosmopoli

disease is common in human populations where poor sanitation and substandard personal hygiene prevail. The high prevalence areas include tropical and subtropical regions, like Mexico, Central and South America, India, South East Asia, Eastern and South Africa (Wells and Arguedas 2004). The transmission of amoebiasis is via fecal–oral route through the ingestion of infective stage cysts. Severity of the disease ranges from asymptomatic carrier to intestinal amoebiasis with symptoms that include amoebic colitis and dysentery, and the potentially fatal extra-intestinal amoebiasis caused by the haematogenous spread of active multiplying trophozoites to other organs. Amoebic liver abscess (ALA) is the most common manifestation of extra-intestinal amoebiasis (Petri and Singh 1999). Delay in diagnosis and subsequent treatment are the common causes of fatality in ALA cases (Akgun et al. 1999).

During active infection, *E. histolytica* trophozoites secrete and/or excrete products into the host environment. These excretory and secretory (ES) products contain virulence factors like amoebapores, cysteine proteases, collagenases, glycosidases, and other proteases that had been hypothesized to contribute to the pathogenesis of *E. histolytica* (Gitler et al. 1984; Guerrero-Manriquez et al. 1998; Debnath et al. 2005; Moncada et al. 2005). In addition, these ES products had also been shown to possess antigenic properties which are useful for diagnostic applications (Pal et al. 1996; Gupta et al. 1999; Sengupta et al. 2000). In order to collect the ES products, a protein-free maintenance medium is necessary to avoid "contamination" with non-parasite proteins and to maintain the viability of trophozoites. In addition, to facilitate reproducibility of the experiments, chemically defined medium would be preferred.

Previous studies have been performed to collect ES products by incubating *E. histolytica* trophozoites in various protein-free media, such as phosphate-buffered saline (PBS), Hank's balanced salt solution (HBSS), Dulbecco's Modified Eagle Medium (DMEM), and serum- and vitamin-free TYI-S-33 (Reed et al. 1989; Gupta et al. 1999; Moncada et al. 2005; Pal et al. 1996; Sengupta et al. 2000). These ES products were collected at the time when the viability of the trophozoites was at least 95%. The results showed that chemically non-defined TYI-S-33 medium was the most suitable to sustain ≥95% viability of trophozoites for up to 7 h. However the data in the above studies lacked statistical analysis to make convincing conclusions. Thus it is necessary that studies on media comparisons using appropriate statistics be performed to determine the best protein-free and chemically defined medium, for collection of *E. histolytica* ES products.

An early study by Diamond (1961) reported that 0.1% of L-cysteine and 0.02% ascorbic acid (C&A) could create an artificial anaerobic environment in axenic media for cultivation of *E. histolytica* (Dutta 1981). In the latter studies by Gillin and Diamond (1980a, b), they have shown that the addition of C&A in maintenance medium containing bovine serum and vitamins can sustain the viable *E. histolytica* for 12–24 h (Martinez-Palomo 1982).

In the present study, four types of protein-free and chemically defined media commonly used in tissue culture were compared to determine the most suitable maintenance medium for sustaining a minimum of 95% viability for axenically grown *E. histolytica* trophozoites. Simultaneously the effect of supplementing the media with C&A was also studied.

Materials and methods

Axenic culture of *Entamoeba histolytica*

The *E. histolytica* HM-1:IMSS axenic strain was used in this study. The trophozoites were hermetically cultured in TYI-S-33 medium, containing 10% heat-inactivated bovine serum (Gibco, New Zealand) and supplemented with 3% Diamond vitamin (Sigma, USA), at 36°C (Diamond et al. 1978).

Preparation of protein-free maintenance media with/without C&A

The four types of media used in this experiment were as follows: PBS for amoeba (PBS-A; 15 mM potassium phosphate and 175 mM sodium chloride, pH 7.0), HBSS without phenol red, pH 7.0, DMEM (Gibco, USA), Roswell Memorial Park Institute medium, No 1640 (RPMI 1640; Gibco, USA). All media were prepared using distilled water. PBS-A was autoclaved and HBSS was filter-sterilized using 0.22-μm filter. In addition, all media with C&A supplementation were also prepared.

Viability study of *Entamoeba histolytica* in different protein-free media over time

A preliminary study was performed to estimate the longest time duration for survival of at least 95% of the trophozoites in the four protein-free media. As the control, trophozoites were maintained in serum- and vitamin-free TYI-S-33 medium, i.e. undefined medium commonly used to grow *E. histolytica*. The morphology of the parasite in each culture medium was observed and recorded until the cells became rounded. For quantitative data analysis, viability of trophozoites was studied in RPMI 1640 media and DMEM for 8 h; and for up to 6 h in PBS-A and HBSS. The duration of incubation was set based on preliminary observations.

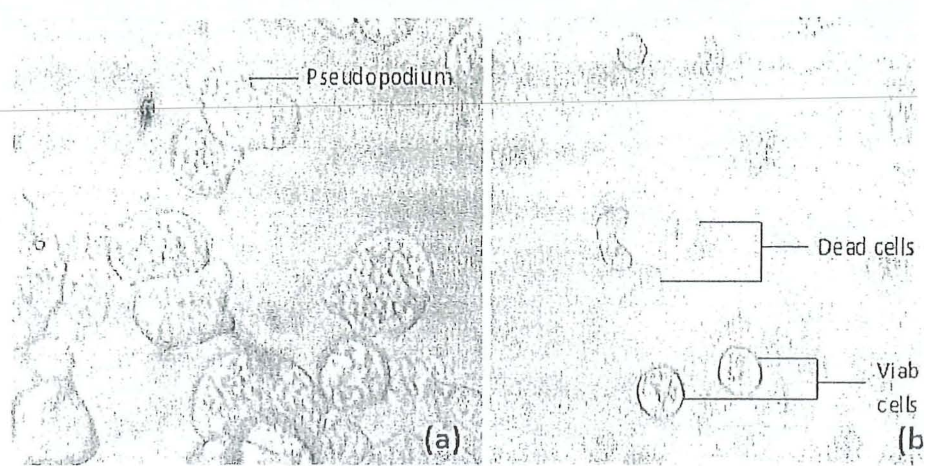


Fig. 1 Morphology of axenic *E. histolytica* trophozoites in PBS-A at magnification of 200× during **a** log phase and **b** after incubation for 6 h

The trophozoites used in the viability study were 48–72 h old, at which time they formed a monolayer on the wall of the culture tube containing the axenic TYI-S-33 medium. At each hourly interval, duplicate amoebic culture tubes containing each type of medium were examined. First, each tube was gently rinsed twice with 5 mL of a protein-free medium. The tube was then filled to the 80% level (10 mL) with the test medium. The initial viability of trophozoites was assumed to be 100% (Jimenez et al. 2004). At 1-h intervals, two tubes from each medium were chilled in crushed ice for 5 min, and then centrifuged at 500×*g* for 2 min. About 9 mL of supernatant was discarded. The pelleted trophozoites were gently mixed, and then the viability determined by Trypan blue exclusion method using Neubauer's chamber. The viability was expressed as mean of three separate experiments, each with two data replicates.

Statistical analysis

Least Significant Difference post hoc test was used to analyze the significant difference at hourly intervals between control medium and the protein-free media. A P value of <0.05 was considered as statistically different between the control media and the protein-free media.

Results

Morphology of *Entamoeba histolytica* after prolonged incubation in protein-free media

In the preliminary study performed using serum- and vitamin-free TYI-S-33 control medium, viable irregular-shaped trophozoites with amoeboid movements and pseudopodia attaching to the surfaces were observed throughout the 8-h incubation period. However, in the other media with or without C&A supplement, the trophozoites started to lose their viable characteristics (as described above) after 3 h. the incubation time in the protein-free media increases, t cells started to become rounded. Figure 1 shows the chang in morphology when PBS-A was used as the medium.

Viability of *Entamoeba histolytica* in different protein-fr medium over time

Figure 2 showed the comparisons among the protein-fr defined media with or without C&A supplementation, ar TYI-S-33 medium (serum- and vitamin-free) as the contr The *E. histolytica* trophozoites in the control mediu consistently showed ≥95% viability throughout th 8-h incubation period. Without C&A supplementation, the end of the 8-h incubation period, DMEM and RPM 1640 showed higher parasite viabilities as compared PBS-A and HBSS. Only RPMI 1640 showed no statistic difference in parasite viability as compared with the contr medium for the first 4 h, however the ≥95% viability wa only maintained at the first 2 h and decreased to 88% at 4 The other protein-free media without the supplemen showed differences in parasite viabilities as compared wi the control media even after 1 h of incubation.

With the addition of C&A supplements, DMEM-C& showed a eightfold increment in parasite viability as com pared to DMEM alone. Both RPMI 1640-C&A and HBSS C&A revealed fourfold and threefold increments, respectiv ly, whereas PBS-A-C&A showed only onefold improveme as compared to the respective media without C&A. Percen age viabilities of both DMEM-C&A and RPMI 1640-C& were maintained at ≥95% throughout the 8-h incubatio period. However, in HBSS-C&A medium, ≥95% trophozoite viability was sustained for only 6 h; then the percentag viability decreased to about 80%. In PBS-A-C&A, th percentage viability of the trophozoites were sustained about 95% for the first 2-h incubation, but dropped to 87% (significantly lower than control medium, P<0.05) during th next hour.

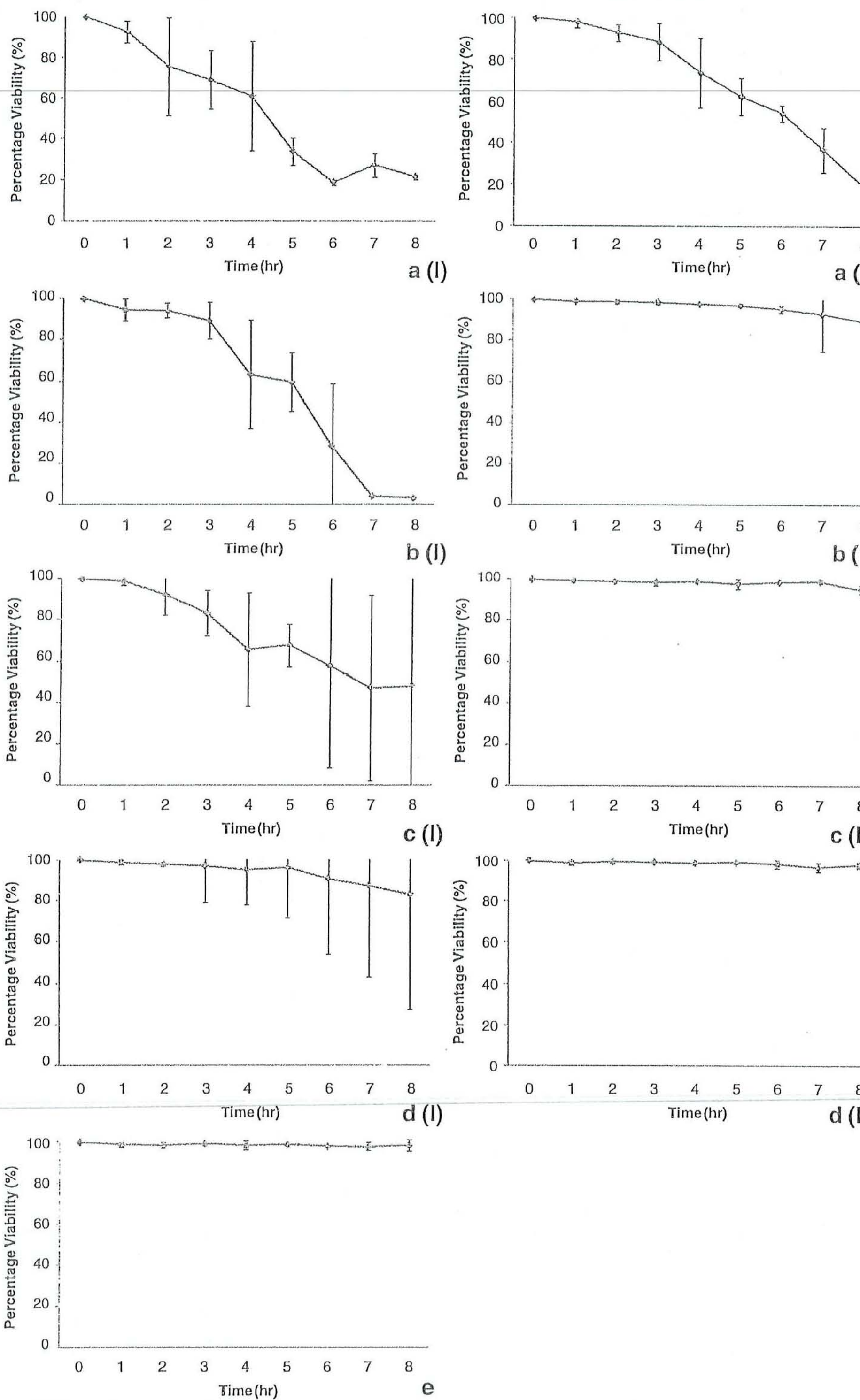


Fig. 2 Comparisons of percentage viabilities of *E. histolytica* trophozoites among the four protein-free media with/without C&A supplementation over time. Serum- and vitamin-free TYI-S-33 was the control medium. **a** (I), PBS-A; **a** (II), PBS-A-C&A; **b** (I), HBSS; **b** (II), HBSS-C&A; **c** (I), DMEM; **c** (II), DMEM-C&A; **d** (I), RPMI 1640; **d** (II), RPMI 1640-C&A; **e**, serum- and vitamin-free TYI-S-33

Discussion

Current ES collection methods could not exclude most of the proteins released from the trophozoites that lysed during the incubation process. Furthermore, Trypan blue exclusion method could only estimate the viability at the end of th incubation period but could not estimate the trophozoit that lysed during the process. Thus protein-free mediu that can prolong the viability of *E. histolytica* trophozoit is pertinent in studies involving ES products. It will redu

the amount of trophozoite proteins released from dead cells and the non-parasite contaminants from serum supplement. A protein-free and chemically defined medium will also facilitate reproducibility of the experimental data. Protein-free medium was also reported to be useful in immunological testing and functional studies such as interaction of *E. histolytica* with different cell lines (Guy et al. 1991).

In this study, viability of *E. histolytica* trophozoites in four different protein-free and chemically defined media suggested that among the media without supplementation, RPMI 1640 was the most suitable in sustaining ≥95% viability for the first 2 h and showed no statistical difference ($P<0.05$) with the control medium as compared with PBS-A, HBSS, and DMEM. With C&A supplementations, there were improvements in the general viability profiles over time in all media. This was especially evident by extension of the duration of ≥95% viability of trophozoites in PBS-A-C&A (onefold), HBSS-C&A (threefold), DMEM-C&A (eightfold), and RPMI 1640-C&A (fourfold). Similar result was also observed in the axenic culture of *Giardia lamblia*, another anaerobic intestinal protozoan. The RPMI 1640 medium supplemented with L-cysteine was reported to promote the viability and attachment of the parasite after prolonged incubation (Guy et al. 1991).

E. histolytica is an anaerobic protozoan that needs a low oxygen tension environment to grow (Sen et al. 2007). Band and Cirrito (1979) revealed that it was able to tolerate up to only 5% oxygen in culture media. In order to create an artificial anaerobic media for its survival, supplementation of 0.1% L-cysteine and 0.02% ascorbic acid into the axenic growth media (TYI-S-33 and TP-S-1) was introduced by Diamond (1961). With these supplements, the media was able to support the survival, as well as growth of *E. histolytica* trophozoites. Other reducing agents such as D-cysteine and thioglycolic acid have also been included into *E. histolytica* trophozoites culture for the same purpose (Gillin and Diamond 1980b). However, the combination of C&A was found to be the best for growth of trophozoites culture (Martinez-Palomo 1982). These supplements were reported to act as reducing agent as well as protective agent against oxidative stress. Later studies reported that good growth of trophozoites was also achieved using reducing agents like 0.2% L-cysteine and 0.2% reduced glutathione (Tekwani and Mehlotra 1999). However, thus far these supplements have not commonly been used in culture media.

Results from the present study were consistent with the earlier study by Gillin and Diamond (1980a, b), in which the maintenance medium supplemented with C&A was able to sustain the attachment, elongation, and amoeboid movement, as well as short-term survival of *E. histolytica* trophozoites. Similar report on cysteine supplementation in PBS (0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 20 [illegible] cysteine) showed that it could sustain ≥95% viability [illegible] trophozoites for 3–4 h (Reed et al. 1989). This sugge[illegible] that adding L-cysteine in PBS could improve the viabilit[illegible] trophozoites even in the absence of ascorbic acid.

There were obvious variations in the trophoz[illegible] viability periods when cultured in different media. In [illegible] study, ≥95% trophozoite viability was observed in PB[illegible] for less than 1 h, which is less than the 2 h reported [illegible] Sengupta et al. (2000) and 4 h reported by Gupta et [illegible] (1999). In comparison to the results by Sengupta et [illegible] (2000), the present study showed that HBSS sustained [illegible] same viability duration (2 h); but RPMI 1640 sustained [illegible] hour longer than the results in the former study.

In conclusion, among the four protein-free media used [illegible] this study, RPMI 1640 and HBSS could sustain ≥9[illegible] trophozoite viability for up to 2 h, hence are not suitable [illegible] maintenance media for prolonged incubation. However, ≥9[illegible] trophozoite viabilities were prolonged to 8 h with b[illegible] DMEM-C&A and RPMI 1640-C&A; and 6 h with HB[illegible] C&A. In conclusion, this study showed that either DMEM [illegible] RPMI 1640 media supplemented with C&A were suitable [illegible] ES production since they could sustain ≥95% trophozo[illegible] viability for up to 8 h.

Acknowledgements This study was supported by Universiti Sa[illegible] Malaysia Research University grant, no. 1001/PPSK/813009, FR[illegible] grant, no. 203/CIPPM/6711122, and USM-RU-PRGS no. 10[illegible] INFORMM/8032030. The first and second authors received financ[illegible] support from USM Fellowship program.

References

Akgun Y, Tacyildiz IH, Celik Y (1999) Amebic liver absces[illegible] changing trends over 20 years. World J Surg 23:102–106

Band RN, Cirrito H (1979) Growth response of axenic *Entamoe[illegible] histolytica* to hydrogen, carbon dioxide, and oxygen. J Protozo[illegible] 26:282–286

Debnath A, Akbar MA, Mazumder A, Kumar S, Das P (2005) *Entamoe[illegible] histolytica*: characterization of human collagen type I and Ca[illegible] activated differentially expressed genes. Exp Parasitol 110:21[illegible]–219

Diamond L (1961) Axenic cultivation of *Entamoeba histolytic[illegible]* Science 134: 336–337

Dutta GP (1981) Experimental and clinical studies on amoebias[illegible] McGraw-Hill, New Delhi

Gillin FD, Diamond LS (1980a) Attachment and short-term maint[illegible]nance of motility and viability of *Entamoeba histolytica* in [illegible] defined medium. J Protozool 27:220–225

Gillin FD, Diamond LS (1980b) Attachment of *Entamoeba histolyti[illegible]* to glass in a defined maintenance medium: specific requireme[illegible] for cysteine and ascorbic acid. J Protozool 27:474–478

Gitler C, Calef E, Rosenberg I (1984) Cytopathogenicity of *Entamoe[illegible] histolytica*. Philos Trans R Soc Lond B Biol Sci 307:73–85

Guerrero-Manriquez GG, Sanchez-Ibarra F, Avila EE (1998) Inhib[illegible]tion of *Entamoeba histolytica* proteolytic activity by huma[illegible] salivary IgA antibodies. APMIS 106:1088–1094

Gupta S, Naik S, Naik SR (1999) Vaccine potential of 56–66 kDa protease secreted by *Entamoeba histolytica*. Indian J Med Res 109:141–146

Guy RA, Bertrand S, Faubert GM (1991) Modification of RPMI 1640 for use in vitro immunological studies of host-parasite interactions in giardiasis. J Clin Microbiol 29:627–629

Jimenez JC, Fontaine J, Grzych JM, Dei-Cas E, Capron M (2004) Systemic and mucosal responses to oral administration of excretory and secretory antigens from *Giardia intestinalis*. Clin Diagn Lab Immunol 11:152–160

Martinez-Palomo A (1982) The biology of *Entamoeba histolytica*. Research Studies Press, Chichester

Moncada D, Keller K, Chadee K (2005) *Entamoeba histolytica*-secreted products degrade colonic mucin oligosaccharides. Infect Immun 73:3790–3793

Pal S, Sengupta K, Manna B, Sarkar S, Bhattacharya S, Das P (1996) Comparative evaluation of somatic & excretory-secretory antigens of *Entamoeba histolytica* in serodiagnosis of human amoebiasis by ELISA. Indian J Med Res 104:152–156

Petri WA Jr, Singh U (1999) Diagnosis and management of ameb Clin Infect Dis 29:1117–1125

Que X, Reed SL (2000) Cysteine proteinases and the pathogenes amebiasis. Clin Microbiol Rev 13:196–206

Reed SL, Keene WE, McKerrow JH (1989) Thiol protei expression and pathogenicity of *Entamoeba histolytica*. J Microbiol 27:2772–2777

Sen A, Chatterjee NS, Akbar MA, Nandi N, Das P (2007) The kilodalton thiol-dependent peroxidase of *Entamoeba histol* is a factor involved in pathogenesis and survival of the pa during oxidative stress. Eukaryot Cell 6:664–673

Sengupta S, Akbar A, Mukhopadhyay P, Ganguly S, Sen P, D (2000) Role of excretory-secretory products of *Entam histolytica* in human amebiasis. Arch Med Res 31(4 Su S226–S228

Tekwani BL, Mehlotra RK (1999) Molecular basis of defence ag oxidative stress in *Entamoeba histolytica* and *Giardia lam* Microbes Infect 1:385–394

Wells CD, Arguedas M (2004) Amebic liver abscess. S Med J 97:673-

Identification of *Entamoeba histolytica* trophozoites in fresh stool sample: comparison of three staining techniques and study on the viability period of the trophozoites

Tan, Z.N.[1], Wong, W.K.[3], Nik Zairi, Z.[2], Abdullah, B.[2], Rahmah, N.[3], Zeehaida, M.[2], Rumaizi, S.[4], Lalitha, P.[5], Tan, G.C.[2], Olivos-Garcia, A.[6] & Lim, B.H.[1]
[1] School of Health Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia.
[2] Department of Medical Microbiology and Parasitology, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia
[3] Institute for Research in Molecular Medicine, Universiti Sains Malaysia, 11800, Penang, Malaysia.
[4] Laboratory Animal Research Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia.
[5] Faculty of Applied Sciences, AIMST University, 08000 Sungai Petani, Kedah, Malaysia.
[6] Departamento de Medicina Experimental, Facultad de Medicina, Universidad Nacional Autónoma de México, 04510 México D.F., México
Corresponding author e-mail: limbh493@gmail.com
Received 16 December 2009; received in revised form 13 January 2010; accepted 15 January 2010

Abstract. *Entamoeba histolytica* causes about 50 million infections worldwide with a death rate of over 100,000 annually. In endemic developing countries where resources are limited, microscopic examinations based on Wheatley trichrome staining is commonly used for diagnosis of intestinal amoebiasis. Other than being a time-consuming method, it must be performed promptly after stool collection as trophozoites disintegrate rapidly in faeces. The aim of this study was to compare the efficacies of Eosin-Y, Wheatley trichrome and Iodine stains in delineating the diagnostic features of the parasite, and subsequently to determine the suitable microscopy observation period for detection of erythrophagocytic and non-erythrophagocytic trophozoites spiked in semi-solid stool sample. Wheatley trichrome staining technique was performed using the standard method while the other two techniques were performed on the slides by mixing the respective staining solution with the spiked stool sample. One million of axenically cultured non-erythrophagocytic *E. histolytica* and erythrophagocytic *E. histolytica* were separately spiked into 2 g of fresh semi-solid faeces. Percentage viability of the trophozoites in the spiked stool sample was determined at 30 minute intervals for eight hours using the 0.4% Trypan blue exclusion method. The results showed that Eosin-Y and Wheatley trichrome stained the karyosome and chromatin granules better as compared to Iodine stain. The percentage viability of non-erythrophagocytic trophozoites decreased faster than the erythrophagocytic form in the first 5 hours and both dropped to ~10% in the 6th hour spiked sample. In conclusion, Eosin-Y staining technique was found to be the easiest to perform, most rapid and as accurate as the commonly used Wheatley trichrome technique; Eosin-Y stained slide sealed with DPX could also be kept as a permanent record. A period not exceeding 6 hours after stool collection was found to be the most suitable in order to obtain good microscopy results of viable trophozoites.

INTRODUCTION

Entamoeba histolytica is an enteric anaerobic protozoan parasite that causes about 50 million infections with a death rate of over 100 000 worldwide annually (WHO, 1997; Jackson, 1998; Zlobl, 2001; Fotedar *et al.*, 2007). The amoebic infection is the third most common cause of death among parasitic diseases, after malaria and schistosomiasis (Tanyuksel & Petri, 2003). The disease is widely reported in developing countries like India and Bangladesh, tropical African countries and in some areas in Brazil and Mexico. The incidence is increasing in non-endemic and developed countries such

as the USA and European countries, due to the ease of world travel and immigration of people from endemic areas (Nari *et al.*, 2008). High risk people are those who travel to crowded endemic areas with low standards of hygiene and sanitation; and those who practice unnatural sexual activities such as direct anal-genital and/or oral-anal sex (Espinosa-Cantellano & Martinez-Palomo, 2000; Haque *et al.*, 2000; Zlobl, 2001; Fotedar *et al.*, 2007).

Entamoeba histolytica has a simple life cycle, in which the transmission is via the faecal-oral route. Infection occurs through ingestion of infective cysts (size 8-20 µm) or invasion of motile trophozoites (size 20-40 µm) (Martinez-Palomo, 1982; Lucas & Upcroft, 2001). The infection causes a variety of clinical presentations, from asymptomatic colonization to invasive amoebic dysentery and extraintestinal amoebiasis. Most infected individuals do not show clinical signs, and the problem is compounded by the lack of reliable and practical diagnostic tools (Martinez-Palomo, 1982; Huston *et al.*, 1999; Zlobl, 2001; Blessmann *et al.*, 2003; Huston, 2004; Fotedar *et al.*, 2007).

The routine diagnosis of amoebic dysentery is still based on identification of erythrophagocytic trophozoites in dysenteric specimens (Cheesbrough, 2005). This low- cost diagnostic technique is still the preferred method in developing countries although numerous molecular-based methods such as polymerase chain reaction and immunological-based methods such as enzyme-linked immunosorbent assay, have been reported to be effective in species-specific diagnosis of *E. histolytica* (Huston *et al.*, 1999; Tanyuksel & Petri, 2003; Visser *et al.*, 2006; Fotedar *et al.*, 2007). A major setback in microscopy is the requirement of freshly collected stool samples as the trophozoites had been reported to disintegrate in faeces from 30 minutes to 3 hours after collection (Gardner *et al.*, 1980; Tanyuksel & Petri, 2003; Fotedar *et al.*, 2007); nevertheless there is no conclusive published data to support this claim. Another disadvantage of microscopy is the time consuming Wheatley trichrome staining process, which requires at least 42 minutes to perform (Flournoy *et al.*, 1982).

As amoebiasis mostly occurs in resource-tight developing countries, microscopy technique will still remain the diagnostic method of choice. Laboratories worldwide reportedly used numerous successful staining methods such as Wheatley's trichrome, Iron hematoxylin, Giemsa, Wright's, Methylene blue, Chorazole Black E and Iodine-trichrome stains (Koontz & Weinstock, 1996; Tanyuksel & Petri, 2003; Fotedar *et al.*, 2007), but all are tedious and time-consuming. Hence, a simple, rapid and reliable staining technique is urgently needed. The objectives of this study were to compare the efficacies of Eosin-Y, Wheatley trichrome and Iodine in staining the characteristic features of the parasite; and subsequently to determine the most suitable microscopy observation period for detection of erythrophagocytic and non-erythrophagocytic trophozoites spiked in semi-solid stool sample.

MATERIALS AND METHODS

Staining of trophozoites

Approximately one million *E. histolytica* axenically cultured in TYI-S-33 medium was washed with 1X Phosphate Buffered Saline (PBS) and spiked in 2 g of fresh semi-solid stool sample obtained from a healthy volunteer. Then, spiked stool samples were stained separately with Wheatley trichrome, Iodine and Eosin-Y solution alcoholic with phloxine B (Sigma HT110316, USA) (Eosin-Y). Duplicate slide smears were prepared for each staining technique. Wheatley trichrome staining technique was performed based on the standard operating protocol (SOP) used at the Department of Medical Microbiology and Parasitology, School of Medical Sciences, Universiti Sains Malaysia, Malaysia. An applicator stick was used to smear ~2 mg of stool sample on a clean slide. The smeared slide was then immersed in Schauddin's fixative for 2 hours. This was followed by soaking the slide in succession in tincture of iodine, 70% alcohol, Wheatley

trichrome stain (REMEL Inc., Lenexa, USA), acid alcohol, absolute alcohol and xylene. Finally, the slide was mounted with dibutyl phthalate xylene (DPX) and observed under a light microscope at 1000X magnification. The iodine stained smeared slide was prepared based on the protocol suggested by Koontz & Weinstock (1996). Briefly, an applicator stick was used to mix ~2 mg of stool sample with ~30 µL Lugol's Iodine solution on a clean slide. A cover slip was placed on the sample and sealed with DPX, then observed under a microscope. In Eosin-Y staining technique, an applicator stick was used to mix ~30 µL of Eosin-Y with ~2 mg of stool sample on a clean slide. A cover slip placed over the sample was sealed with DPX, then observed under a microscope at 400X and 1000X magnification. The images of the trophozoites stained by all three methods were captured using an Olympus Image Analysis System (Olympus System Microscope Model BX41, Japan). Comparisons were made among the images of the three types of stained trophozoites based on the clarity of their characteristic nuclear features.

Viability of non-erythrophagocytic and erythrophagocytic trophozoites in stool sample

About one million cultured trophozoites washed with 1X PBS were spiked into 2 g of fresh semi-solid stool sample. About 2 mg of the sample was mixed with 50 µl Trypan (0.4%) blue and the mixture was loaded into a Neubauer chamber to determine the viability of non-erythrophagocytic trophozoites by microscopy. The procedure was performed in duplicate and repeated every 30 minute intervals for eight hours.

In order to determine the viability of erythrophagocytic trophozoites, about 10 µL of blood was first added into a sterile microfuge tube containing 1 x 10^6 axenically cultured trophozoites. After 30 minutes, the trophozoites were washed with 1X PBS and spiked into 2 g of fresh semi-solid stool sample. Then, ~2 mg spiked stool sample was mixed with 0.4% Trypan blue, and the percentage viability was determined as described earlier.

Direct wet mounts were also prepared to observe the movement of motile trophozoites and their disintegration over time. An applicator stick was used to mix ~2 mg of spiked stool sample with ~30 µL normal saline (0.85% NaCl) on a clean slide. Then, a cover slip was placed on the sample and observed immediately under a light microscope.

RESULTS AND DISCUSSION

Staining of trophozoites

Images of the trophozoites were compared based on the detection of the characteristic features of trophozoites such as the chromatin granules that line the nuclear membrane and the small spherical karyosome at the centre of the nucleus. Nucleus of trophozoite has no fixed position in the cytoplasm, but moves freely and sometimes rotates rapidly (Martinez-Palomo, 1982). Thus, observation of the characteristic features of live trophozoites requires fine focusing of the optical microscope at 400X or 1000X magnification.

Permanent stains were much more effective than the direct wet mount for detection of trophozoites and/or cysts in stool specimens (Gardner *et al.*, 1980). Figure 1(a) shows the image of a Wheatley trichrome stained trophozoite; it was stained blue-purple with greenish background, with good delineation of the chromatin granules and karyosome. The stain provided a good contrast between the trophozoite and the background debris. However, an obvious disadvantage was the tedious protocol which required 2 hours fixation period and a total time of ~3 hours to complete. Appropriate fixation periods coupled with sufficient washing steps are pertinent in obtaining a well-stained nucleus, thus may require the preparation of a number of slides for each stool sample. Repeated use of acid alcohol in destaining trichrome stain will reduce its efficiency and subsequently require a longer destaining time although a better alternative is to use a fresh solution. The suggested fixation time with Schauddin's fixative is between 2 to 24 hours. Any increase in

fixation time must be followed by an appropriate increase in washing time using tincture of iodine. Moreover, Schauddin's fixative, which killed and fixed the trophozoites contains mercury compound which is not environmentally-friendly (Garcia & Shimizu, 1998; Amin, 2000). This staining technique demands technical skills of an experienced microscopist, and would be daunting to those unskilled personnel who have to perform the technique occasionally.

Iodine stain is mostly used to identify *E. histolytica* cysts in stool microscopic detection (Cheesbrough, 2005). However, Koontz & Weinstock (1996) reported that the stain could be used to delineate intestinal amoebas by negating the motility of the trophozoites. As shown in figure 1 (b), the nuclear chromatin granules were only faintly stained and the karyosome remained unstained.

Figures 1(c) and 1(d) show Eosin-Y stained non-erythrophagocytic and erythrophagocytic trophozoites, respectively. The former shows a trophozoite with its well-stained nuclear chromatin granules and karyosome; and the latter reveals well-stained characteristic features of the erythrophagocytic amoebic trophozoite and the engulfed erythrocytes. The whole trophozoite was stained light red, and both the chromatin granules and karyosome showed distinctly dark appearances. Eosin-Y also clearly stained the engulfed erythrocytes.

Various types of eosin stains are available commercially and some are used as counterstain to haematoxylin in Haematoxylin and Eosin (H&E) stain. Its acidic property stains the basic components of a cell, such as cytoplasm, light red in colour. Others used eosin as an exclusion dye to stain dead trophozoites light red in colour to distinguish them from the unstained viable trophozoites (Mirelman *et al.*, 1987; Behnia *et al.*, 2008). The stain was also reportedly used to facilitate the detection of motile trophozoites by staining the background pink without staining the live parasites (Cheesbrough, 2005). Interestingly, phloxine B in Eosin-Y was reportedly used to stain nuclear structures in histological sections (SPI.Supplies, 2009). Until now, there were no reports on the use of phloxine B to stain nucleus of *E. histolytica*. Here, we showed that Eosin-Y was just as accurate as Wheatley trichrome staining method in identification of trophozoites in stool samples. Besides staining the characteristic nuclear features of the trophozoites and/or the engulfed erythrocytes, it could be performed easily to give spontaneous results. The Eosin-Y used in this study is commercially available in its working dilution, thus can be applied directly onto the stool samples without fuss. Alternatively, Eosin-Y staining solution can also be prepared by mixing 1% (w/v) Eosin-Y, 1% (w/v) phloxine B, 95% ethanol and glacial acetic acid in appropriate volumes (Mayer's, 2009).

Another major advantage of Eosin-Y staining technique is that the stained trophozoites could easily be visualized under 400X magnification. At this magnification, it is very difficult to identify the Wheatley trichrome stained trophozoites. The rounded shape and immotile trophozoites left for 3 hours in stool sample were also easily stained by Eosin-Y [Figure 1(e)]. In fact, this stain was able to preserve the general morphology of the trophozoite for more than 24 hours. This was probably due to the presence of alcohol and glacial acetic acid in the stain, as these two chemicals are also used in Schauddin's fixative.

A major advantage of Wheatley trichrome staining technique is that it be used to prepare a permanent record of the stained amoebas. In contrast, the stained nuclear chromatin granules and karyosome of an Eosin-Y stained trophozoite gradually became fainter over time and almost indistinguishable from its cytoplasm after an hour [Figure 1(f)]. However, by sealing the edges of the cover slip to the slide with DPX, it prevented Eosin-Y from drying. This permanent record of the Eosin-Y stained slide could be stored longer if placed in a horizontal position (instead of a vertical position).

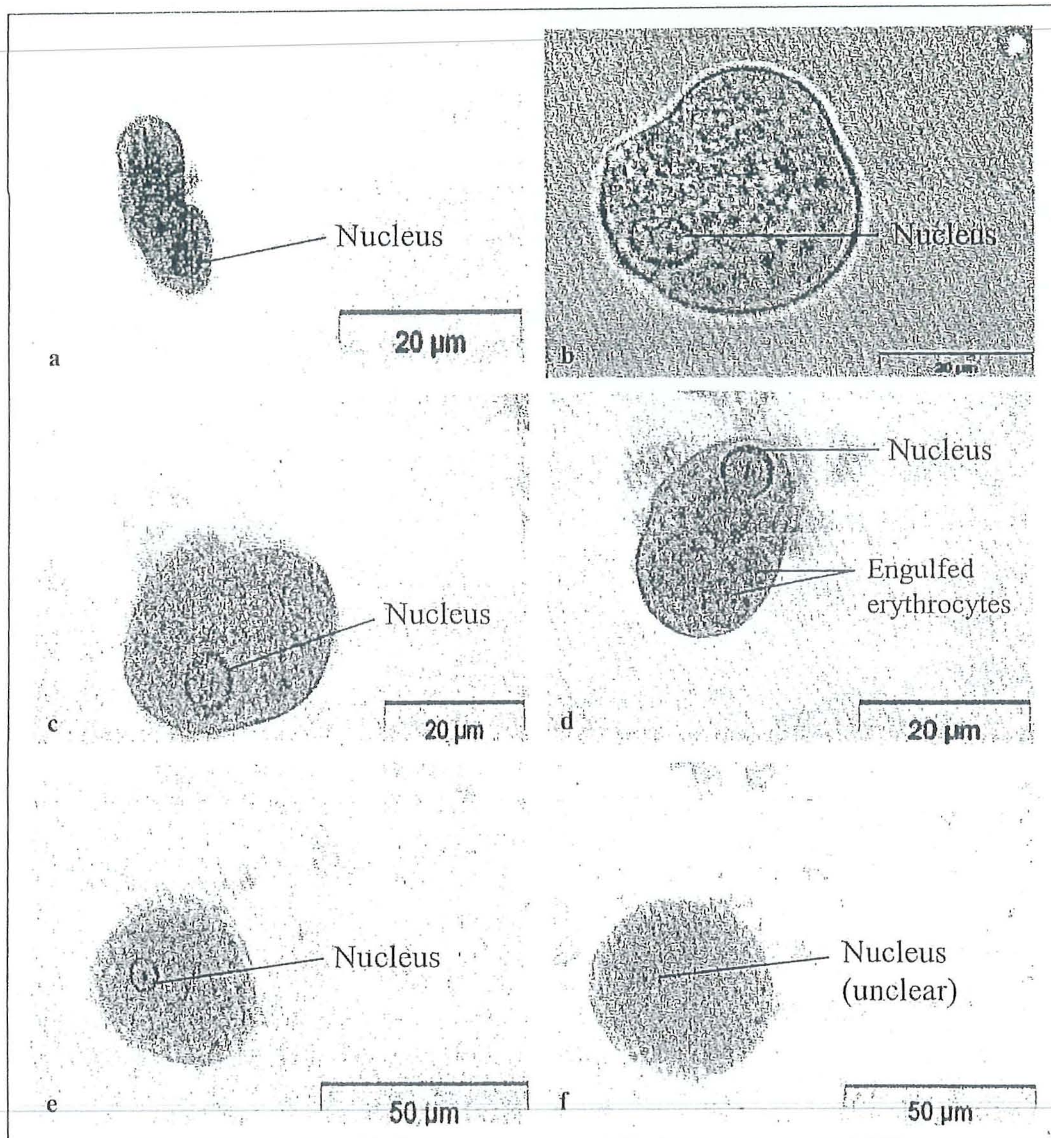


Figure 1. Stained trophozoites. (a) Wheatley trichrome stained trophozoite, 1000X magnification (b) Iodine stained trophozoite, 1000X magnification (c) Eosin-Y stained trophozoite, 1000X magnification (d) Eosin-Y stained erythrophagocytic trophozoite, 1000X magnification (e) Eosin-Y stained trophozoite showed clear chromatin granules and karyosome, 400X magnification (f) Eosin-Y stained trophozoite without DPX seal indicated unclear nuclear characteristics after an hour, 400X magnification.

Viability of non-erythrophagocytic and erythrophagocytic *E. histolytica* trophozoites in stool sample

Gonzalez-Ruiz *et al.* (1994) reported that trophozoites started to disintegrate rapidly as soon as they were in the faeces. However, the viability period of trophozoites outside its host was not studied. In the present study, the viability of trophozoites in stool sample was assessed by Trypan blue dye exclusion test whereby the dead trophozoites were stained blue and the live ones remained unstained (Figure 2). The viability chart of the non-erythrophagocytic trophozoites in spiked semi-solid stool is shown in (Figure 3). During the first hour, the percentage viability dropped rapidly and fluctuated at approximately 55%. This was probably due to the unfavourable conditions in the stool as compared to the optimal axenic

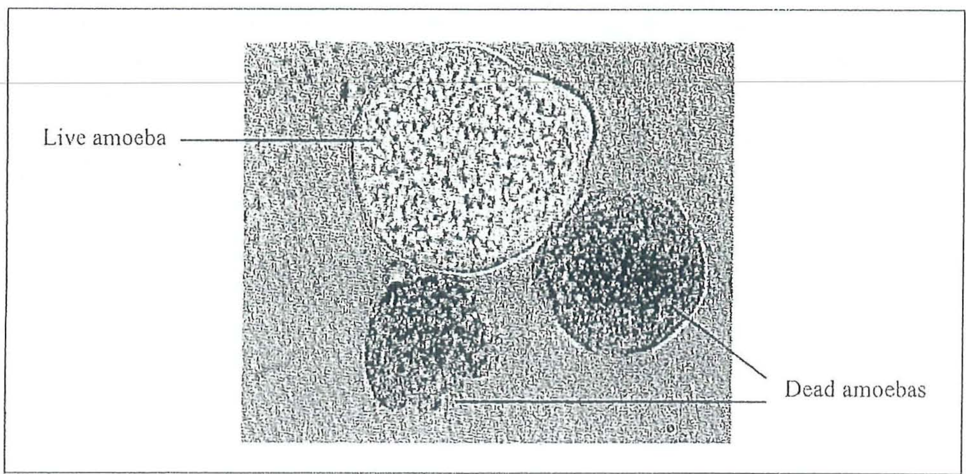


Figure 2. Trypan blue dye exclusion stained trophozoites, 1000X magnification.

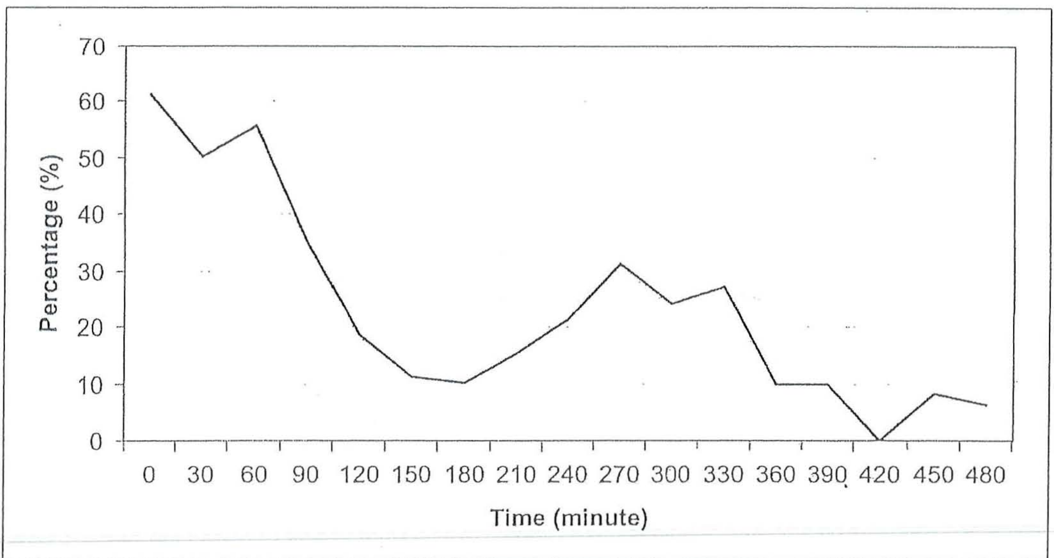


Figure 3. Viability chart of *E. histolytica* trophozoites in spiked semi-solid stool.

conditions of the trophozoites in TYI-S-33 medium at 36°C. Thereafter, the viability dropped to ~10% at the third hour. However, during the 3 to 5½ hours period, the percentage viability increased slightly, and then fluctuated around ~30%. At the 7th hour, none of the trophozoites was detected but ~10% viability was again observed at the 8th hour. This was probably due to the fact that *E. histolytica* in the stool samples was being challenged with a toxic high oxygen environment (30%) since it has been reported that amoebas can be supported in only less than 5% O_2 (Band & Cirrito, 1979). Figure 4 shows the viability chart of the erythrophagocytic trophozoites in spiked semi-solid stool. In comparison with figure 3, the percentages viability of erythrophagocytic trophozoites was higher (65% and 95%) during the first and third hours in stool sample. This was probably due to the antioxidant molecules (superoxide dismutase, catalase, glutathione, peroxirredoxin and vitamin E) present in the

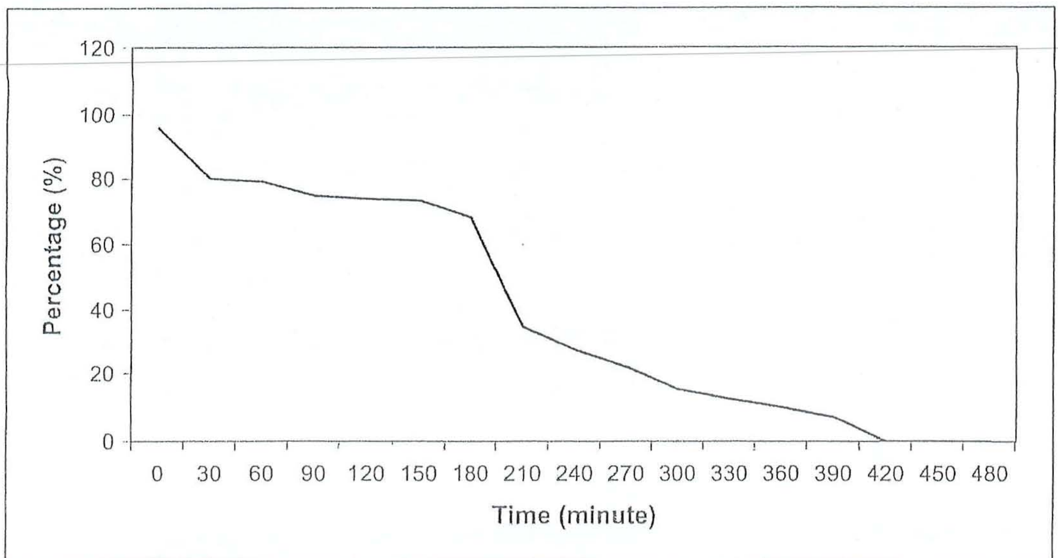


Figure 4. Viability chart of *E. histolytica* erythrophagocytic trophozoites in spiked semi-solid stool.

engulfed erythrocytes (Kuypers, 2007), which helped the amoeba to detoxify the reactive oxygen species generated during the oxygen reduction and/or because the erythrocytes were source of nutrients for the amoebas. Between the third and sixth hours, the mortality of the trophozoites increased gradually from about 65% to 90%, and none was detected from the seventh hour onwards. In general, the percentages viability of both forms of trophozoites dropped to ~10% at the sixth hour in semi-solid stool sample and none was detected from the seventh hour onwards. Since ~90% of the trophozoites were undetected at the sixth hour, microscopy detection to detect the amoebas should thus be performed within six hours after stool collection.

Observation of the direct wet mount slide preparation during the first hour in fresh semi-solid stool sample revealed that the trophozoites did not have fixed shape and were actively pushing out the ectoplasm to form pseudopodia, followed by the in-flowing endoplasm. In addition, the technique allowed the disintegration process of trophozoites to be observed over time (Figure 5a). Uroid of the amoeba was located at the posterior end of the live trophozoites. Faint engulfed erythrocytes were also visible but the characteristic nucleus was impossible to visualize without staining (Figure 5b). Thus the direct wet mount technique is neither sensitive nor reliable for detection of *E. histolytica* in stool samples.

All microscopy staining techniques (include those used in this study) cannot differentiate *E. histolytica* from the non-pathogenic *E. dispar*. However parasite identification by staining is still commonly used in developing endemic countries where resource are limited, as the costs of commercially available *E. histolytica* antigen detection tests are prohibitive. An important supportive evidence for microscopy is the detection of erythrophagocytic trophozoites in stool sample, although some non-pathogenic *Entamoeba* species may also ingest erythrocytes (Gonzalez-Ruiz *et al.*, 1994). Indiscriminate use of antiparasitic drugs may lead to development of drug-resistant. Thus, treatment should only be given to patients where the presence of *E. histolytica* in stool is confirmed, and no treatment should be administered if only *E. dispar* is found (WHO, 1997). Until now, light microscopic differentiation between the two amoeba species is not yet available and WHO has highlighted the urgent need in developing improved methods for the species-specific diagnosis of *E. histolytica* infection (WHO,

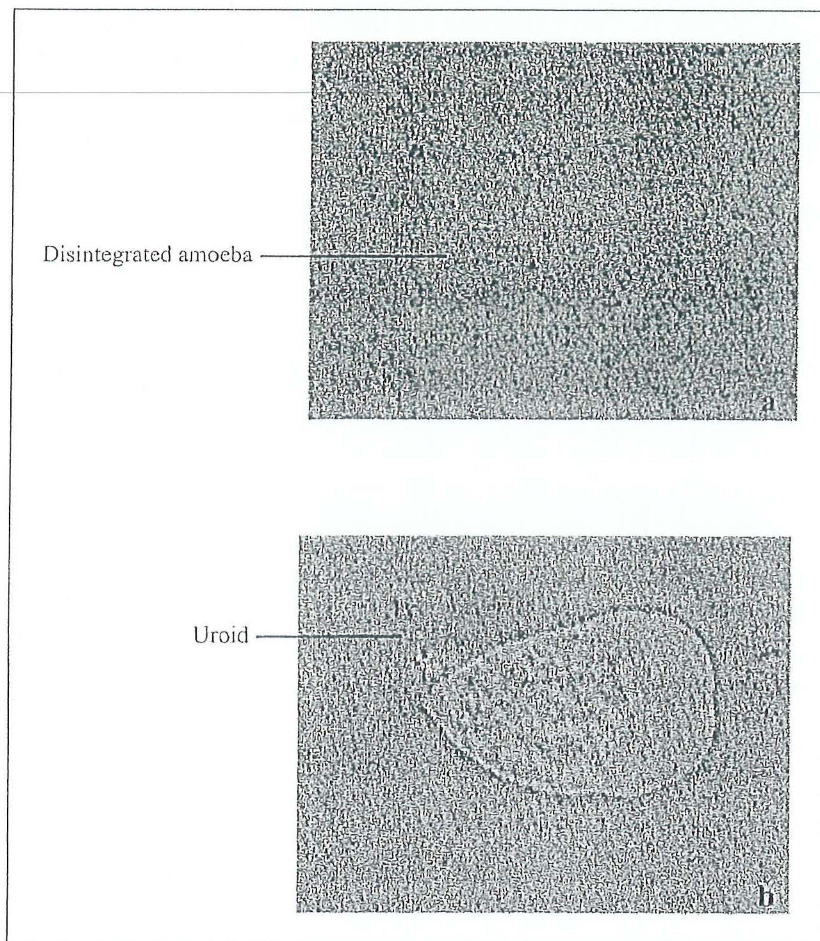


Figure 5. (a) Disintegrated trophozoite, 1000X magnification (b) Motile trophozoite with uroid at posterior end examined using direct wet mount, 1000X magnification.

1997). Hence efforts should also be on the search for stains which can specifically demonstrate structural compounds found in *E. histolytica* but absent in *E. dispar*.

In conclusion, this study showed that for microscopic identification of *E. histolytica* in patients' samples, Eosin-Y could stain the characteristic nuclear chromatin granules and karyosome of the trophozoites as accurately as the Wheatley trichrome, and better than the Iodine stain. Eosin-Y stained slide could also be kept as permanent record if the cover slip is sealed to the slide with DPX, however further studies are needed to determine the period of time before drying occurs. Nevertheless, Eosin-Y technique offers the added advantages of being rapid and easy to perform, thus is very useful for the purpose of identification of *E. histolytica* in patients' stool samples, especially in busy, and/or understaffed laboratories. The identification of *E. histolytica* was supported by the signs and symptoms presented by the patients and the detection of erythrophagocytic trophozoites. This study also suggests that the microscopy observation for viable trophozoites is best performed within the first 6 hours after stool collection.

Acknowledgement. This study was funded by a research university grant from Universiti Sains Malaysia (1001/PPSK/813009). The first author received financial support through

the university fellowship. Special thanks to Mohd Khairul Afif Azman for his technical assistance and Zainul Fadziruddin Zainuddin for proofreading the manuscript.

REFERENCES

Amin, O.M. (2000). Evaluation of a new system for the fixation, concentration, and staining of intestinal parasites in fecal specimens, with critical observations on the trichrome stain. *Journal of Microbiological Methods* **39:** 127-132.

Band, R.N. &, Cirrito, H. (1979). Growth response of axenic *Entamoeba histolytica* to hydrogen, carbon dioxide, and oxygen. *Journal Protozoology* **2:** 282-286.

Behnia, M., Haghighi, A., Komeylizadeh, H., Tabaei, S.J. & Abadi, A. (2008). Inhibitory effects of Iranian Thymus vulgaris extracts on *in vitro* growth of *Entamoeba histolytica*. *Korean Journal of Parasitology* **46:** 153-156.

Blessmann, J., Ali, I.K., Nu, P.A., Dinh, B.T., Viet, T.Q., Van, A.L., Clark, C.G. & Tannich, E. (2003). Longitudinal study of intestinal *Entamoeba histolytica* infections in asymptomatic adult carriers. *Journal of Clinical Micro-biology* **41:** 4745-4750.

Cheesbrough, M. (2005). *District Laboratory Practice in Tropical Countries.* 2nd Edition. Cambridge University Press. pp. 200-202.

Espinosa-Cantellano, M. & Martinez-Palomo, A. (2000). Pathogenesis of intestinal amebiasis: from molecules to disease. *Clinical Microbiology Reviews* **13:** 318-31.

Flournoy, D.J., McNabb, S.J., Dodd, E.D. & Shaffer, M.H. (1982). Rapid trichrome stain. *Journal of Clinical Microbiology* **16:** 573-574.

Fotedar, R., Stark, D., Beebe, N., Marriott, D., Ellis, J. & Harkness, J. (2007). Laboratory diagnostic techniques for *Entamoeba* species. *Clinical Micro-biology Reviews* **20:** 511-532.

Garcia, L.S. & Shimizu, R.Y. (1998). Evaluation of intestinal protozoan morphology in human fecal specimens preserved in EcoFix: comparison of Wheatley's trichrome stain and EcoStain. *Journal of Clinical Microbiology* **36:** 1974-1976.

Gardner, B.B., Del Junco, D.J., Fenn, J. & Hengesbaugh, J.H. (1980). Comparison of direct wet mount and trichrome staining techniques for detecting *Entamoeba* species trophozoites in stools. *Journal of Clinical Microbiology* **12:** 656-658.

Gonzalez-Ruiz, A., Haque, R., Aguirre, A., Castanon, G., Hall, A., Guhl, F., Ruiz-Palacios, G., Miles, M.A. & Warhurst, D.C. (1994). Value of microscopy in the diagnosis of dysentery associated with invasive *Entamoeba histolytica*. *Journal of Clinical Pathology* **47:** 236-239.

Haque, R., Mollah, N.U., Ali, I.K., Alam, K., Eubanks, A., Lyerly, D. & Petri, W.A., Jr. (2000). Diagnosis of amebic liver abscess and intestinal infection with the TechLab *Entamoeba histolytica* II antigen detection and antibody tests. *Journal of Clinical Microbiology* **38:** 3235-3239.

Huston, C.D. (2004). Parasite and host contributions to the pathogenesis of amebic colitis. *Trends in Parasitology* **20:** 23-26.

Huston, C.D., Haque, R. & Petri, W.A., Jr. (1999). Molecular-based diagnosis of *Entamoeba histolytica* infection. *Expert Reviews in Molecular Medicine* **1999:** 1-11.

Jackson, T.F. (1998). *Entamoeba histolytica* and *Entamoeba dispar* are distinct species; clinical, epidemiological and serological evidence. *International Journal for Parasitology* **28:** 181-186.

Koontz, F. & Weinstock, J.V. (1996). The approach to stool examination for parasites. *Gastroenterology Clinics of North America* **25:** 435-449.

Kuypers, F.A. (2007). Membrane lipid alterations in hemoglobinopathies. *Hematology American Society of Hematology Education Program* 68-73.

Lucas, R. & Upcroft, J.A. (2001). Clinical significance of the redefinition of the agent of amoebiasis. *Revista Latinoamericana de Microbiologia* **43**: 183-187.

Martinez-Palomo, A. (1982). *The Biology of Entamoeba histolytica*. Research Studies Press. A Division of John Wiley & Sons Ltd. pp. 1-59.

Mayer's. (2009). H & E staning Method and Protocol [Online] [Accessed 14/10/ 2009 9:19AM], Available from World Wide Web: http://www.ihcworld.com/_protocols/ special_stains/HE_ Mayer.html

Mirelman, D., Monheit, D. & Varon, S. (1987). Inhibition of growth of *Entamoeba histolytica* by allicin, the active principle of garlic extract (Allium sativum). *Journal of Infectious Diseases* **156**: 243-244.

Nari, G.A., Ceballos Espinosa, R., Carrera Ladron de Guevara, S., Preciado Vargas, J., Cruz Valenciano, J.L., Briones Rivas, J.L., Moreno Hernandez, F. & Gongora Ortega, J. (2008). [Amebic liver abscess. Three years experience]. *Revista Española de Enfermedades Digestivas* **100**: 268-272.

SPI.Supplies. (2009). [Online] [Accessed 13/ 10/2009 5:18 PM], Available from World Wide Web: http://www.2spi.com/com/ catalog/chem/Phloxine_B.shtml

Tanyuksel, M. & Petri, W.A. (2003). Laboratory diagnosis of amebiasis. *Clinical Microbiology Reviews* **16**: 713-729.

Visser, L.G., Verweij, J.J., Van Esbroeck, M., Edeling, W.M., Clerinx, J. & Polderman, A.M. (2006). Diagnostic methods for differentiation of *Entamoeba histolytica* and *Entamoeba dispar* in carriers: performance and clinical implications in a non-endemic setting. *International Journal of Medical Microbiology* **296**: 397-403.

WHO. (1997). Amoebiasis. *WHO Weekly Epidemiological Record* **72**: 97-100.

Zlobl, T.L. (2001). Amebiasis. *Primary Care Update for OB/GYNS* **8**: 65-68.

Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage:www.elsevier.com/locate/apjtb

Document heading

Detection of *Entamoeba histolytica* in experimentally induced amoebi liver abscess: comparison of three staining methods

Tan Zi Ning[1], Wong Weng Kin[2], Shaymoli Mustafa[1], Arefuddin Ahmed[1], Rahmah Noordin[2], Tan Gim Cheon Olivos–Garcia Alfonso[4], Lim Boon Huat[1*]

[1]*School of Health Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia*
[2]*Institute for Research in Molecular Medicine, Universiti Sains Malaysia, 11800 Penang, Malaysia*
[3]*Department of Medical Microbiology and Parasitology, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia*
[4]*Departamento de Medicina Experimental, Facultad de Medicina, Universidad Nacional Autónoma de México, 04510 México D.F., México*

ARTICLE INFO

Article history:
Received 28 May 2011
Received in revised form 27 June 2011
Accepted 13 July 2011
Available online 28 January 2012

Keywords:
Entamoeba histolytica
Amoebic liver abscess
H&E
PAS
IHC
Trophozoite

ABSTRACT

Objective: To compare the efficacy of three different tissue stains, namely haematoxylin ar eosin (H&E), periodic–acid Schiff (PAS) and immunohistochemical (IHC) stains for detectic of *Entamoeba histolytica* (*E. histolytica*) trophozoites in abscessed liver tissues of hamste **Methods:** Amoebic liver abscess was experimentally induced in a hamster by injecting 1 × 1 of axenically cultured virulent *E. histolytica* trophozoites (HM1–IMSS strain) into the portal vei. After a week post–inoculation, the hamster was sacrificed and the liver tissue sections we stained with H&E, PAS and IHC stains to detect the amoebic trophozoite. **Results:** The thre stains revealed tissue necrosis and amoebic trophozoites, but with varying clarity. H&E and PA stained the trophozoites pink and magenta, respectively, however it was difficult to differentia the stained trophozoites from the macrophages because of their similarity in size and morpholog On the other hand, IHC stain revealed distinct brown appearance of the trophozoites in th infected liver tissues. **Conclusions:** It can be concluded that out of the three stains, IHC is th best for identification of *E. histolytica* trophozoites in tissue sections.

1. Introduction

Amoebic liver abscess (ALA) is the most common clinical presentation of extraintestinal infection of the intestinal protozoon, *Entamoeba histolytica* (*E. histolytica*). This illness is prevalent worldwide and endemic in tropical countries such as India, Bangladesh, tropical African countries, some areas in Brazil and Mexico, China and South–east Asia. Although less than 1% of patients infected with *E. histolytica* develop ALA, this still represents an alarming number. The ailment is easily acquired in poor sanitation area, via ingestion of infective *E. histolytica* cysts present in contaminated hands, food or water. Interestingly, the incidence rate is also increasingly reported in non- endemic and developed countries such as USA an European countries because of the ease of world travel an immigration of people from endemic areas[1–3].

Pathogenesis of ALA is known to be very complicated It develops through the hematological dissemination o the pathogenic trophozoites into liver via the tributaries o portal vein after invasion of colonic mucosa, resulting in th formation of solitary or multiple abscesses regularly foun in the right liver lobe[4]. The common virulence factor involved include Gal/GalNAc specific lectin, cystein proteinases, amoebapores and lipophosphopeptidoglyca molecules[5,6]. In the formation of ALA, the genera sequence of morphological changes in liver tissues involve acute inflammation where the acute cellular infiltration i composed of polymorphonuclear leukocytes which surroun the centrally located amoebas, then progress to granulom formation after the leukocytes were being replaced b macrophages and epithelioid cells and subsequentl

*Corresponding author: Lim Boon Huat, School of Health Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia.
Tel: [illegible]
Fax: [illegible]
E-mail: [illegible]

Foundation Project: Supported by a grant from Universiti Sains Malaysia grant No. [illegible]

Several kinds of laboratory animal models have reportedly been used to study the formation of ALA. Since 1950s, inoculation routes such as direct intrahepatic, intracaecal, intraperitoneal and intraportal were performed to induce ALA in hamster, mouse and gerbil. Currently, the intraportal injection of *E. histolytica* trophozoites in hamster has been widely used to produce ALA[9,10] and this technique is adopted in the present study.

A good staining method is pertinent in the pathogenesis study on ALA. An excellent stain facilitates visualization of the morphological changes in liver tissues and also differentiates the amoebas against surrounding cells such as hepatocytes, macrophages and other cell types[11]. The staining techniques reportedly used are haematoxylin and eosin (H&E), periodic–acid Schiff (PAS) and immunostaining. However, comparison of the efficiency of these staining methods in detecting amoebas has not been reported. Thus, this study was aimed to compare the efficacy of H&E, PAS and immunohistochemical (IHC) stains for detection of *E. histolytica* trophozoites in liver tissue of hamster with ALA.

2. Materials and methods

2.1. Development of ALA in experimentally induced hamster

ALA was induced in a Syrian golden hamster as described by Olivos–Garcia and Weber *et al*[12,13]. Briefly, 1×10^{6} of axenically cultured virulent strain *E. histolytica* trophozoites (HM1–IMSS) was suspended in 0.2 mL phosphate buffer saline (PBS) and then inoculated into the portal vein of an anesthetized male hamster. After one week post–inoculation, the animal was sacrificed with a three–time overdose of pentobarbital. Immediately after the animal became unconscious, cardiac puncture was performed to collect the blood, then transferred into a sterile 1.5 mL microfuge tube and allowed to clot. The hamster serum containing polyclonal antibody against *E. histolytica* was then stored at –20 ℃ until used. The liver was removed aseptically, followed by fixation in 10% formalin. The same procedures were performed in the control healthy hamster, except that the injection fluid comprised 0.2 mL PBS. The animal experimentation was approved by USM Animal Research Ethics Committee [No. Animal Ethics Approval: USM/Animal Ethics Approval/2008/(40)(129)].

2.2. Tissue processing

Both infected and healthy formalin–fixed livers were cut into small pieces and kept in separate cassettes. The tissues were then processed overnight in an automated tissue processer (Leica TP 1020, Germany), which involved a total of 6 h, followed by 3 h clearing with xylene and 4 h tissue impregnation with embedding medium. The processed liver tissues were then embedded in paraffin wax to produce tissue blocks. Four μm thick formalin-fixed, paraffin–embedded tissue sections were cut with a microtome (Microm HM 325 Rotary Microtome, Germany) and subsequently stained with the three stains. Triplicate tissue sections were prepared for each stain.

2.3. Histochemical staining methods

2.3.1. H&E stain

Staining of the processed tissue sections was performed according to the standard protocol as described by Bancroft and Gamble with some modifications[14]. In brief, processed tissues were deparaffinized with two changes of xylene for 2 min each, rehydrated with two changes of absolute, 95% and 80% alcohols for 2 min each, followed by washing in running tap water for 5 min. Then, the tissues were stained with Harris's haematoxylin (Sigma–Aldrich, USA) for 20 min and washed in running tap water. Differentiation with 1% acid alcohol was carried out for 10 sec, followed by washing and bluing by dipping the tissues in ammonia water for 10 sec. After a washing step, the tissues were counterstained with eosin Y (Sigma–Aldrich, USA) for 2 min, dehydrated with increasing graded of alcohols for 2 min each, cleared with two changes of xylene for 2 min each and finally mounted with dibutyl phthalate xylene (DPX).

2.3.2. PAS stain

Slides were prepared based on the conventional protocol described by Bancroft and Gamble[14]. Briefly, processed tissues underwent the same deparaffinization, rehydration and washing steps as mentioned in the H&E stain. Next, the tissues were treated with periodic acid solution (Sigma–Aldrich, USA) for 5 min and washed with distilled water for 5 min. The tissues were then covered with Schiff's reagents for 10 min, followed by washing in running tap water for 5 min. Counterstaining was performed with Harris's haematoxylin (Sigma–Aldrich, USA) for 1 min, then washed in running tap water for 5 min and differentiated with 1% acid alcohol. Subsequently, the tissues were dipped in ammonia water for 10 sec until the sample turned blue, washed in running tap water for 5 min, followed by dehydration with increasing graded of alcohols, cleared with xylene and mounted with DPX.

2.4. Immunohistochemical staining method (IHC stain)

Indirect staining was performed on processed tissue sections with some modifications of the standard protocol as described by Bancroft and Gamble[14]. First, the tissues

were deparaffinized with two changes of xylene for 5 min each, followed by rehydration with two changes of absolute, 70% and 50% alcohols for 3 min each and washing in running tap water for 5 min. Tissues were then blocked with 3% hydrogen peroxide for 5 min, dipped in distilled water for 5 min and followed by 30 min incubation with 1:100 dilution of the corresponding polyclonal hamster serum sample *i.e.* sera from the ALA–induced hamster and control hamster used for the infected and control tissues, respectively. Washing steps were then carried out five times with PBS–Tween 20 (PBST), 2 min each. Tissues were incubated with 1:1 000 dilution of HRP–conjugated anti–hamster antibody (Sigma–Aldrich, USA) for 30 min and again washed with PBST. After washing, the tissues were developed with 3,3′–diaminobenzidine (DAB) substrate solution for 3 min and again washed with PBST. Finally, the tissues were counterstained with Harris's haematoxylin (Sigma–Aldrich, USA) for 1 min, followed by washing, differentiation with 1% acid alcohol, bluing with ammonia water, another washing step, dehydration with increasing graded alcohols, clearance with xylene and then mounted with DPX.

Finally, the three differently stained tissues were observed under a light microscope at different magnifications (40×, 100× and 400×) and the images were captured using image analysis system (Nikon eclipse 80i, Japan). Comparisons on the ease and clarity of *E. histolytica* trophozoites detection were then made based on the captured images.

3. Results

Gross examinations of both the infected and non–infected liver tissues were performed prior to processing for histology. The infected liver was found to be enlarged and studded with multiple small yellow–white abscesses, whereas the non–infected liver was normal in size with a smooth clean surface (Figure 1). All the triplicate stained tissue slides revealed similar overall appearance. The healthy liver tissue sections revealed intact hepatic lobules with central veins and cords of radiating hepatocytes surrounded by the portal triads. On the contrary, in sections from infected liver tissue, a well defined endothelial layer of central vein was not observed as seen in normal tissue section (Figure 2). The abscesses in the infected tissue were seen as foci of extensive necrolysis and degenerative changes. Efficacy of each staining method was compared in terms of the ease and clarity of trophozoites detection from tissue sections. With H&E stain, the trophozoites were stained pink whereas the PAS stain outlined the trophozoites magenta in colour. Both the stains could not differentiate the trophozoites clearly, as the amoebas resembled the macrophages. However, with the immunostain, the trophozoites were stained brown in colour, an end–product of the enzymatic reaction between DAB and horseradish peroxidase. Consequently, the appearances of IHC–stained trophozoites were easily identified from the background of inflamed and necrotic tissues (Figure 3). In Figure 4, the images captured from IHC stained slides clearly revealed central necrotic region in liver tissue surrounded by scanty inflammatory cells with amoebic trophozoites along the margins. Islands of better preserved liver tissue were also seen scattered among the necrotic foci.

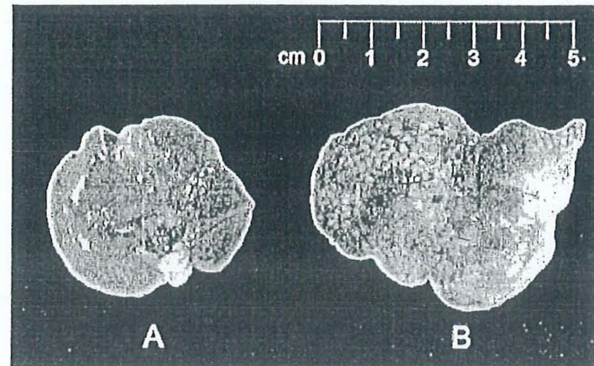


Figure 1. Gross appearance of hamster livers.
A: Non–infected healthy liver with a smooth and clean surface; B: One–week post inoculation abscessed liver with multiple tiny whitish spots.

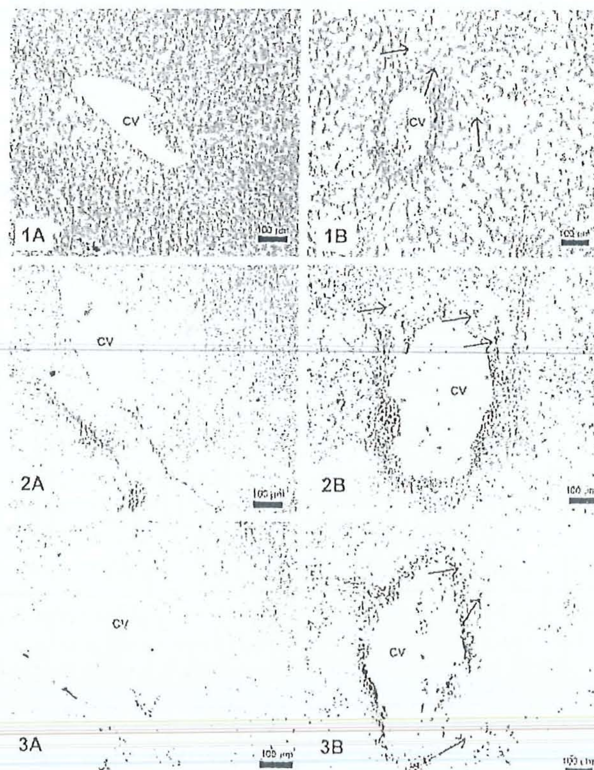


Figure 2. Photomicrographs showing normal liver tissues (left) and infected liver tissues (right) using three different staining techniques. 1A, 1B: H&E stain; 2A, 2B: PAS stain; 3A, 3B: IHC stain (100×); CV: Central vein. *E. histolytica* trophozoites are indicated with arrows. All the sections from non–infected liver show normal liver architecture with intact central vein and cords of hepatocytes. Sections from the infected liver show necrolytic tissues with distorted central vein.

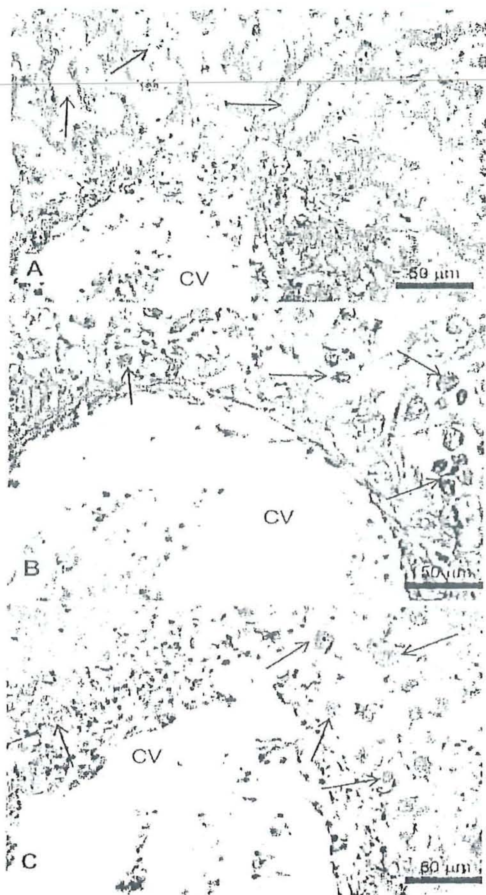


Figure 3. Micrographs indicating the different staining of *E. histolytica* trophozoites.
A: H&E stain; B: PAS stain; C: IHC stain (400×); CV: Central vein.
E. histolytica trophozoites are indicated with arrows. (A) Trophozoites (arrow) are visible as round, oval to pear shaped cells lying in lacunar spaces with occasional ingested red blood cells inside, very similar to macrophages in morphology. (B) PAS stained section showing the trophozoites (arrow) with magenta coloured cell membrane in a necrotic background. (C) IHC stain showing brown coloured trophozoites (arrow) with a distinct cell membrane easily identifiable against a background of necrosis and inflammation.

Figure 4. Photomicrograph from IHC stained liver tissue from infected hamster showing extensive necrosis representing coalescing microabscesses.
E. histolytica trophozoites (brownish, marked with arrow) are seen along the abscess margins invading the better preserved liver tissue. Magnification: 40×. N: necrotic area; LC: well-preserved liver cells.

4. Discussion

ALA has been known to be a potentially fatal extraintestin infection of amoebiasis. Multiple factors involving parasi and the host have been reported to be involved in tl development of ALA. The general concept of developme of ALA involves the adaptation and survival of amoeb in liver tissue[9]. Rigothier *et al*[11], reported that the was massive death of parasites after a few hours of posi infection and inflammation in the hamster liver tissue wa caused. After 12 h, the parasites started multiplying and th size of inflammation foci increased. In addition, other facto such as oxygen reduction ability, complement resistant, RO and NOS scavenger capacity and immune evasion of th parasites also contribute to the parasites survival. Once th parasites are able to adapt to the environment in the live inflammation will be stimulated and followed by extensiv tissue destruction[8,15,16].

In this study, the results showed that tissue destructio and amoebae in the tissue sections can be visualized b all the three stains, but with varying clarity. H&E and PA stains required high technical expertise to identify an interpret the staining results. Even though H&E stain i the most widely employed histology stain to demonstrat the morphology of different cells and tissue[14], it has bee reported to be not ideal for detection of amoebic trophozoite especially in the examination of fixed and stained biops samples due to the difficulty in differentiating the staine trophozoites from the surrounding tissues. PAS stains tissu carbohydrates magenta, and it is commonly used to stai liver glycogen[14]. The problem arises because *E. histolytic* trophozoites are also magenta in colour when stained wit PAS, possibly due to the presence of glycoprotein in th amoeba cell membrane[17]. Thus, with both the H&E and PA stains, amoebic trophozoites were difficult to differentiat from macrophages because of their similarities in size an morphology[18].

In comparison, IHC is presumed to be more specific as i is the consequence of specific reactions between antigen of amoebic trophozoite and antibodies against them. I this study, immunostaining gave more distinct and easil identifiable appearance of the trophozoites in a backgroun of necrosis and inflammation as compared with the othe two staining techniques. Even though numerous reporte studies on amoebic pathogenesis utilized H&E and PA stains, this study showed that IHC stain was more superio than the two stains. As was previously described for hamste and human ALA[9,19], the images captured from IHC staine slides clearly revealed central necrotic region in liver tissu surrounded by scanty inflammatory cells with amoebi trophozoites along the margins. Islands of better preserve liver tissue were also seen scattered among the necroti foci. Moreover, serum sample could easily be obtaine from 5–7 days post–infected hamster, and containe sufficient polyclonal antibodies that recognize *E. histolytic* trophozoites[20].

A previous study has reported that monoclonal antibod can be used in cryopreserved tissue section to stain amoeba but not in formalin–fixed, paraffin–embedded tissue[21] However, this study showed that amoeba in paraffin–

embedded tissue can be visualized when polyclonal antibody was employed. The use of polyclonal antibody may be able to show stronger antigen recognition on amoebas in the formalin fixed tissue sections as compared with monoclonal antibodies which only recognize single epitopes. Also, processed tissue is favored to cryopreserve tissue because the structures of amoeba are physically supported by the embedding medium, while amoebic structure might be lost with frozen treatment due to the water crystallization.

Nowadays, in the diagnosis of amoebiasis, stool, blood, liver pus, urine and saliva samples are often investigated with various molecular-based and immunological-based techniques[22-25], whereas staining techniques are hardly reported. However, IHC is still relevant for confirmation of numerous pathogenic diseases[14], but rarely reported for use in the investigation of invasive amoebiasis. Thus, it is potentially important as a confirmatory test for ALA if sample from aspiration of liver abscess, liver biopsy or autopsy is available.

In conclusion, in this study, IHC stain was found to be more superior than H&E and PAS stains for detection of *E. histolytica* trophozoites in the infected tissues because the IHC allowed easy identification of brown-stained amoebas among the inflamed and necrotic liver cells.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

This study was funded by a research university grant from Universiti Sains Malaysia (1001/PPSK/813009). The first two authors received financial support through the USM Fellowship.

References

[1] Rao S, Solaymani-Mohammadi S, Petri WA, Parker SK. Hepatic amebiasis: a reminder of the complications. *Curr Opin Pediatr* 2009; **21**(1): 145-149.

[2] Parija SC. *Textbook of medical parasitology protozoology & helminthology*. 3rd ed. New Delhi: All India Publishers & Distributors; 2008.

[3] Seeto RK, Rockey DC. Amebic liver abscess: epidemiology, clinical features, and outcome. *West J Med* 1999; **170**(2): 104-109.

[4] Salles JM, Moraes LA, Salles MC. Hepatic amebiasis. *Braz J Infect Dis* 2003; **7**(2): 96-110.

[5] Ackers JP, Mirelman D. Progress in research on *Entamoeba histolytica* pathogenesis. *Curr Opin Microbiol* 2006; **9**(4): 367-373.

[6] Baxt LA, Singh U. New insights into *Entamoeba histolytica* pathogenesis. *Curr Opin Infect Dis* 2008; **21**(5): 489-494.

[7] Costa CA, Fonseca TH, Oliveira FM, Santos JF, Gomes MA, Caliari MV. Influence of inflammation on parasitism and area of experimental amoebic liver abscess: an immunohistochemical and morphometric study. *Parasit Vectors* 2011; **4**(1): 27.

[8] Guo X, Houpt E, Petri WA. Crosstalk at the initial encounter: interplay between host defense and ameba survival strategies. *Curr Opin Immunol* 2007; **19**(4): 376-384.

[9] Santi-Rocca J, Rigothier MC, Guillen N. Host-mic interactions and defense mechanisms in the developme amoebic liver abscesses. *Clin Microbiol Rev* 2009; **22**(1): 65-7.

[10] Tsutsumi V, Shibayama M. Experimental amebiasis: a sel review of some *in vivo* models. *Arch Med Res* 2006; **37**(2): 210-

[11] Rigothier MC, Khun H, Tavares P, Cardona A, Huerre M, Gu N. Fate of *Entamoeba histolytica* during establishme amoebic liver abscess analyzed by quantitative radioimaging histology. *Infect Immun* 2002; **70**(6): 3208-3215.

[12] Olivos-Garcia A, Nequiz-Avendano M, Tello E, Martinez Gonzalez-Canto A, Lopez-Vancell R, et al. Inflammat complement, ischemia and amoebic survival in a experimental amoebic liver abscesses in hamsters. *Exp Pathol* 2004; **77**(1): 66-71.

[13] Weber C, Blazquez S, Marion S, Ausseur C, Vats D, Krzemi M, et al. Bioinformatics and functional analysis of an *Entam histolytica* mannosyltransferase necessary for para complement resistance and hepatical infection. *PLoS Negl T Dis* 2008; **2**(2): e165.

[14] Bancroft JD, Gamble M. *Theory and practice of histologi techniaques*. 5th ed. China: Churchill Livingstone; 2002.

[15] Olivos-Garcia A, Saavedra E, Ramos-Martinez E, Nequiz Perez-Tamayo R. Molecular nature of virulence in *Entamo histolytica*. *Infect Genet Evol* 2009; **9**(6): 1033-1037.

[16] Wong-Baeza I, Alcantara-Hernandez M, Mancilla-Herrera Ramirez-Saldivar I, Arriaga-Pizano L, Ferat-Osorio E, et al. T role of lipopeptidophosphoglycan in the immune response *Entamoeba histolytica*. *J Biomed Biotechnol* 2010; **2010**: 254521.

[17] Aley SB, Scott WA, Cohn ZA. Plasma membrane of *Entamoe histolytica*. *J Exp Med* 1980; **152**(2): 391-404.

[18] Kumar V, Abbas AK, Fausto N, Aster JC. *Robbins & Cotra pathologic basis of disease*. 8th ed. Philadelphia: Saunde Elsevier; 2010.

[19] Costa CA, Nunes AC, Ferreira AJ, Gomes MA, Caliari MV *Entamoeba histolytica* and *E. dispar* trophozoites in the liver hamsters: *in vivo* binding of antibodies and complement. *Paras Vectors* 2010; **3**(1): 23.

[20] Compton SR, Riley LK. Detection of infectious agents in laborator rodents: traditional and molecular techniques. *Comp Med* 200 **51**(2): 113-119.

[21] Sherchand JB, Thammapalerd N, Riganti M, Tharavan S, Punpoowong B. Monoclonal antibody-base immunohistochemical demonstration of *Entamoeba histolytic* in liver tissues of experimentally infected hamster (*Mesocricet auratus*). *Int J Parasitol* 1994; **24**(6): 909-916.

[22] Haque R, Kabir M, Noor Z, Rahman SM, Mondal D, Alam F, et a Diagnosis of amebic liver abscess and amebic colitis by detectio of *Entamoeba histolytica* DNA in blood, urine, and saliva by real-time PCR assay. *J Clin Microbiol* 2010; **48**(8): 2798-2801.

[23] Khairnar K, Parija SC. Detection of *Entamoeba histolytica* DN in the saliva of amoebic liver abscess patients who received pri treatment with metronidazole. *J Health Popul Nutr* 2008; **26**(4 418-425.

[24] Parija SC, Khairnar K. Detection of excretory *Entamoeb histolytica* DNA in the urine, and detection of *E. histolytica* DN and lectin antigen in the liver abscess pus for the diagnosis o amoebic liver abscess. *BMC Microbiol* 2007; **7**: 41.

[25] Othman N, Mohamed Z, Verweij JJ, Huat LB, Olivos-Garcia A Yeng C, et al. Application of real-time polymerase chain reactio in detection of *Entamoeba histolytica* in pus aspirates of live abscess patients. *Foodborne Pathog Dis* 2010; **7**(6): 637-641.

Analysis of *Entamoeba histolytica* excretory-secretory antigen (ESA) and identification of a new potential diagnostic marker

Running title: *Entamoeba histolytica* ESA of diagnostic potential

Wong Weng Kin,[1] Tan Zi Ning,[2] Nurulhasanah Othman,[1] Lim Boon Huat,[2] Zeehaida Mohamed,[3] Alfonso Olivos Garcia,[4] and Rahmah Noordin[1*]

Institute for Research in Molecular Medicine, Universiti Sains Malaysia, 11800, Penang Malaysia[1],

School of Health Sciences, Universiti Sains Malaysia, 16150, Kelantan, Malaysia[2],

Department of Medical Microbiology and Parasitology, School of Medical Sciences, Universiti Sains Malaysia, 16150, Kelantan, Malaysia[3],

Departmento de Medicina Experimental, Facultad de Medicina, Universidad Nacional Autónoma de México 04510 Mexico D.F., Mexico[4]

*Corresponding author. Mailing address: Institute for Research in Molecular Medicine, Universiti Sains Malaysia, 11800, Penang, Malaysia. Phone: 604-6534802. Fax: 604-6534813. E-mail: rahmah8485@gmail.com.

ABSTRACT

Serodiagnosis of amoebiasis remains the preferred method for diagnosis of ALA. However, the commercially available kits are problematic in endemic areas due to the persistent high background antibody titers. Human serum samples (n=38) from patients with amoebic liver abscess (ALA) who live in endemic areas were collected from Hospital Universiti Sains Malaysia during the period of 2008-2010. Western blot analysis using excretory-secretory antigen (ESA) collected from axenically grown *E. histolytica* were probed with the above serum samples. Seven antigenic proteins of ESA with varying reactivities were identified *i.e.* 152 kDa, 131 kDa, 123 kDa, 110 kDa, 100 kDa, 82 kDa and 76 kDa. However, only 152 kDa and 110 kDa proteins showed sensitivities above 80 % in the Western blot analysis. All the antigenic proteins showed undetectable cross-reactivity when probed with healthy human serum samples (n=30) and serum samples from other infections (n=33). From the MALDI-TOF-TOF analysis, the proteins were identified as heavy subunit of *E. histolytica* lectin and *E. histolytica* pyruvate phosphate dikinase, respectively. Use of the *E. histolytica* lectin for diagnosis of ALA has been well reported by researchers and is being used in commercialized kits. However, this is the first report on the potential use of pyruvate phosphate dikinase for diagnosis of ALA, thus this molecule merits further evaluation on its diagnostic value using a larger panel of serum samples.

40 ## INTRODUCTION

41

42 Amoebiasis is caused by the enteric protozoan *Entamoeba histolytica*, which
43 affects 50 million of the world population and leads to 100,000 fatal cases annually (17,
44 18). Amoebic liver abscess (ALA) is the most common clinical manifestation of
45 extraintestinal amoebiasis. It is due to the haematogenous spread of the *E. histolytica*
46 trophozoites from intestine to liver through the portal vein. Patients with ALA present
47 with hepatomegaly, right upper quadrant pain, tenderness of the liver, fever, jaundice and
48 nausea. It may lead to fatal outcome if early diagnosis and treatment are not sought (1,
49 10).

50

51 Diagnosis of ALA is often initiated with radiology imaging to examine the
52 presence of abscess in the liver. If indicated, aspiration of the sample is performed for
53 culture, DNA detection and/or antigen detection. Absence of bacteria growth in the
54 abscess culture could rule out the possibility of pyogenic liver abscess cases. The
55 definitive diagnosis of ALA is by microscopic observation of trophozoites in the abscess
56 fluid, but the sensitivity of microscopic examination is low as the trophozoites are easily
57 disintegrated and most of them reside at the peripheral margin of the abscess.
58 Nevertheless, it is unethical to perform abscess aspirate close to the peripheral margin of
59 abscess, as this may damage the healthy liver tissues. Many reports showed that DNA
60 and antigen detection-based methods performed on the abscess sample *e.g.* PCR, real-
61 time PCR, Techlab *E. histolytica* II antigen detection ELISA, gave high sensitivity (4, 9,
62 16).

63

64

65 Whenever abscess aspiration cannot be performed due to small abscess size or
66 bad condition of the patient, serological test becomes the preferred choice for diagnosis.
67 The available antigen detection tests such as Techlab *E. histolytica* II ELISA, which
68 detects *E. histolytica* lectin antigen, can be used for diagnosis of acute ALA patient who
69 have not received treatment (21). However often patients who are admitted to the hospital
70 with liver abscess have received treatment prior to investigation for ALA, which
71 significantly reduces the sensitivity of the antigen detection test. Thus antibody detection
72 is currently the most common serological test used to detect ALA, either by indirect
73 haemagglutination assay (IHA) or ELISA. However, these tests mostly use amoebic
74 lysate antigen, and are problematic for diagnosis in endemic area where the background
75 anti-amoebic antibody titer is high. Thus in endemic areas, low specificities of these tests
76 were reported with the low cut-off values as suggested by the manufacturer (20, 22).

77

78 Comparison of crude soluble antigen (CSA) with excretory secretory antigen
79 (ESA) of *E. histolytica* have been shown to demonstrate higher positive detection rate
80 when tested with sera of patients with acute amoebic dysentery and asymptomatic cysts
81 passer, and equal sensitivity for diagnosis of ALA (8, 13). Therefore, in our quest to
82 identify new markers to improve the serodiagnosis of ALA, ESA of *E. histolytica* was
83 produced and analysed by SDS-PAGE, two-dimensional electrophoresis (2-DE) and
84 Western blot. The identities of the potential candidates were then identified by mass-
85 spectrometry.

86

87 ## MATERIALS AND METHODS

88

89 **Maintenance of *E. histolytica* trophozoites.** Axenic strain *E. histolytica*
90 trophozoites HM1:IMSS was hermetically cultured in TYI-S-33 medium supplemented
91 with 12.5 % bovine serum (Invitrogen, New Zealand) and 1X Diamond vitamin Tween-
92 80 (Sigma, USA) at 36 °C. The medium was changed every 48-72 hours (2).

93

94 **Collection and preparation of ESA.** Mass culture of *E. histolytica* trophozoites
95 were collected at log phase and washed 3X with RMPI supplemented with 0.1 % L-
96 cysteine and 0.02 % ascorbic acid (RPMI-C&A) with centrifugation at 22 x g for 2 min,
97 RT. Subsequently, the cell density was determined via trypan blue exclusion method.
98 Trophozoites were seeded into culture tube containing 80 % filled RMPI-C&A medium
99 with cell density of 0.8 x 10^6 cells per mL and incubated at 36 °C for 6 hours. Upon
100 completion, culture tubes were subjected to centrifugation at 22 x g for 2 min at 4 °C.
101 The supernatant in the culture tubes were collected and mixed with 0.5 M iodoacetamide
102 to a final concentration of 1 mM. Next, the supernatant was again subjected to
103 centrifugation at 10, 000 x g for 5 min at 4 °C and filtered through 0.2 μm membrane.
104 Subsequently, ESA in the supernatant was concentrated 1000X using U-Tube
105 concentrator with MWCO of 10 kDa. Cocktail protease inhibitor (Roche Diagnostic,
106 Germany) was then added to the concentrated ESA. The protein concentration of the ESA
107 was estimated using Bradford protein assay (8, 13, 19).

108

109 **Serum samples and ethical approval.** Serum samples in the current study were
110 collected from Hospital Universiti Sains Malaysia during the period of 2008-2010. The
111 procedures of collecting and handling the serum samples were approved by USM Human
112 Ethical Committee (Ref. No.: USMKK/PPP/JEPem[213.3(10)]). Human serum samples
113 included in this study were divided into four groups: (i) Group A: human ALA serum
114 samples (n=24) with consistent clinical symptoms (*i.e.* fever, abdominal/right hepatic
115 chest pain, hepatomegaly, and jaundice) and radiological image, and IHA positive results;
116 (ii) Group B: human ALA serum samples (n=14) from patients whose abscess were
117 positive by real-time PCR for *E. histolytica* DNA and negative by bacterial culture; (iii)
118 Group C: healthy blood donor serum samples which were negative by IHA (n=30); (iv)
119 Group D: serum samples from patients with other infections (n=33) *i.e.* salmonellosis
120 (n=5), shigellosis (n=1), *Escherichia coli* septicaemia (n=2), *Staphylococcus* spp.
121 septicaemia (n=2), *H. pylori* (n=6), pyogenic liver abscess (n=4), *Stenotrophomonas*
122 *maltophilia* septicaemia (n=1), Enteropathogenic *Escherichia coli* (n=1), *Ascaris*
123 *lumbricoides* (n=1), *Klebsiella pneumoniae* (n=1) and toxoplasmosis (n=9). These sera
124 were negative by IHA for amoebiasis.
125
126 **SDS-PAGE.** Protein samples were electrophoretically separated via SDS-PAGE
127 using Bio-Rad Mini Protean III Electrophoresis Cell and Protean® II xi Cell according to
128 Laemmli (7) protocol with modifications. Prior to SDS-PAGE, ESA was mixed with 2X
129 Laemmli sample buffer and boiled for 5 min. Subsequently, it was separated using 6%
130 SDS-PAGE gel, at constant current of 25 mA per gel for about 1 h.
131

132 **Western blotting.** Upon completion of SDS-PAGE, proteins in the gel was
133 electrophoretically transferred onto a 0.45 μm nitrocellulose membrane (NCP) using
134 semi-dry transblot (Bio Rad, USA) at a constant voltage of 15 V for 30 min. The NCP
135 was blocked for 1hr at RT with 5 % skim milk prepared in 10 mM Tris buffered saline,
136 pH 7.2 (TBS). Subsequently, the NCP was washed (3 x 5 min) with TBS containing 0.1
137 % Tween-20 (TBS-T). Then, the NCP was cut into multiple strips and incubated with
138 human sera at dilution of 1:200 (in TBS-T) for 2 hours at RT. The NCP strips were then
139 washed three times with TBS-T, and then incubated with monoclonal mouse anti–human
140 IgG conjugated with horseradish peroxides (HRP) at dilution of 1:6000 for 1hr.
141 Subsequently, the NCP strips were again washed (3 x 5 min) with TBS-T. Western blot
142 substrates *i.e.* enhanced chemiluminescence (ECL) blotting reagent (Roche diagnostics,
143 Germany) or tetramethylbenzidine (TMB) substrate for membrane (Sigma, USA) were
144 usedas substrates. The Western blot signal was captured using camera (Lumix,
145 Germany).
146

147 **2-DE and Western blot.** Selected protein bands which showed potential
148 diagnostic value were further analysed using 2-DE to ensure that the bands were well-
149 separated. OFFGEL fractionator 3100 (Agilent Technologies, Germany) was used to
150 separate the proteins by isoelectric points (pI) followed by SDS-PAGE and Western blot.
151 Agilent OFFGEL Kit pH 3–10 with a 12-well setup frame was used. Sample was
152 prepared by mixing 1600 μl of the ready stock solution (1.25X) with 400 μl of the sample
153 with total protein amount of 2 mg and then gently vortexed. Forty microliter of IPG strip
154 rehydration buffer was added into each well to swell the gel for 15 minutes. Wetted
155 electrode pads were placed at the cathode- and anode-ends of the IPG strip gel surface.
156 After re-swelling of the gel, 150 μL of protein sample was loaded into each well. Ten
157 microliter of rehydration buffer was reapplied onto the electrode pads at each of the IPG
158 gel ends. Cover fluid (mineral oil) was pipetted onto the gel strip ends. Subsequently, the
159 sample was focused with a maximum power of 200 mW, maximum current of 50 mA and
160 typical voltages ranging from 500 to 4500 V until 50 kVh was reached after 24 h. Upon
161 completion, each of the twelve fractionated ESA samples were separately mixed with 5X
162 Laemmli sample buffer without boiling and electrophoretically separated via SDS-
163 PAGE.Western blot was performed using pooled and individual human serum samples to
164 identify the selected antigenic proteins.
165
166 **Mass spectrometry analysis & protein Identification.** The selected proteins
167 were excised from 2D-SDS-PAGE gel and sent for MALDI-TOF-TOF (4800) analysis at
168 Proteomic Laboratory Service Center, Australia, and searched with Swiss-Prot protein
169 database.

RESULTS

IgG blots of ESA. IgG blots of ESA probed with human ALA serum samples from Group A showed seven antigenic bands with consistent reactivities (Fig.1). Besides, these antigenic proteins were also similar with the bands present in the IgG blots probed with human ALA serum samples from Group B (Fig. 2). However, mean sensitivities of the bands to detect ALA vary from 16 % to 84 %. Only two of the antigenic bands *i.e.* 152 kDa and 110 kDa showed high sensitivities of about 80 % in both Groups A and B sera (Table 1). Neither of these two antigenic bands showed reactivity in IgG blots probed with serum samples from Groups C and D, thus showing 100 % specificity.

2-DE Western blot and protein identification. IgG blot of 12 ESA fractions with pooled serum sample revealed that the 152 kDa and 110 kDa proteins were located in Fraction 5 (pI: 5.33-5.91) and Fraction 6 (pI: 5.91-6.5), respectively (Fig. 3). Further IgG blot analysis of these ESA fractions with individual serum samples (n=5) confirmed the location of these antigenic proteins (Fig. 4). These protein bands were excised and sent for MALDI-TOF-TOF analysis. According to Mascot search result from MSDB search engine, the 152 kDa protein matched with *E. histolytica* lectin protein (C4LTMO) with the protein score of 273. A score >55 indicates identity or extensive homology at a significant level ($p < 0.05$). Seven peptides matched to the Gal/GalNAc lectin heavy subunit. The 110 kDa protein matched with pyruvate phosphate dikinase (EHI_009530) with the protein score of 544, with nine matched peptides.

193 **DISCUSSION**

194 Current diagnosis of ALA still depends on the results of clinical manifestations,
195 radiology imaging and laboratory tests, since stool examination is inapplicable for
196 diagnosis of extraintestinal amoebiasis. Detection of *E. histolytica*-specific DNA and
197 antigen in the aspirated abscess are more sensitive and specific, as compared to
198 microscopic examination of live trophozoites. However, in most settings when abscess
199 aspiration cannot be performed, serological test is the alternative laboratory method for
200 diagnosis of ALA. Besides being relatively less invasive, it is also less technical
201 demanding and thus is routinely used in most diagnostic laboratories.

202

203 *E. histolytica* ESA contains proteins shed from trophozoites during active
204 multiplication and metabolites released by trophozoites during incubation in RPMI-C&A.
205 Although great care was taken to produce good quality ESA, there were probably still
206 some partial proteins released from lysed trophozoites.In this study, the ESA antigenic
207 bands that ranged from 97.2-158 kDa consistently showed reactivities when incubated
208 with human ALA serum samples (n=7). Analysis of the IgG blots showed that 152 kDa
209 and 110 kDa proteins had higher association with serum samples from patients with
210 PCR-positive abscess (93 % and 86 %) as compared to serum samples from patients with
211 unknown PCR results (both 79 %). Besides, the sensitivity of both 152 kDa and 110 kDa
212 were similar, *i.e.* 84 % and 82 %, respectively. Both antigens showed high specificity as
213 there were undetectable reactivities in the IgG blot of ESA probed with serum samples
214 from normal individuals and those with other infections.

215

216 The protein components of ESA in the current study were different from those
217 reported by Sengupta *et al.*(13). In the latter study, the ESA proteins ranged between 200-
218 20 kDa, with predominant protein bands below 66 kDa (*e.g.* 45 kDa and 29 kDa), while
219 the high molecular weight proteins (> 100 kDa) were faint. This may be due to the
220 differences in antigen preparations. The ESA in this study was concentrated 1000X
221 instead of 10X to enrich the low abundant proteins. In addition, to enhance the
222 reproducibility of ESA, protein-free defined RPMI-C&A was used in this study, instead
223 of serum- and vitamin-free TYI-S-33. Besides, iodoacetamide was added to the RPMI-
224 C&A containing the ESA upon its collection, in order to protect the protein from
225 degradation by proteases (3, 11, 13).

226

227 2-DE protein separation *via* Agilent 3100 OFFGEL Fractionator followed by
228 SDS-PAGE allowed only the selected ESA fractions to be tested with serum samples.
229 The protein bands excised from the 2-DE gels were well-separated, thus avoiding the
230 presence of multiple proteins in each band. In this study, the 152 kDa protein was
231 identified as *E. histolytica* lectin protein, which has been reported to be sensitive for
232 diagnosis of invasive amoebiasis (5, 6). Specific monoclonal antibody against this protein
233 has been used for antigen detection test in TechLab Entamoeba histolytica II kit
234 (TechLab Inc, USA). The 110 kDa protein, identified as the *E. histolytica* pyruvate
235 phosphate dikinase, was also found to show similar high sensitivity for diagnosis of ALA.
236 This protein was reported to be a key enzyme in the anaerobic metabolism *via*
237 pyrophosphate dependent glycolysis, and has no counterpart with proteins in human
238 metabolism(12). Molecular modeling of this enzyme had been reported, and specific

239 inhibitors to it for therapeutic purpose have been studied (15). The protein sequence of
240 pyruvate phosphate dikinase showed high similarity with a closely related pathogenic
241 intestinal anaerobic protozoa *i.e. Giardia lamblia*; this suggests the possibility of
242 producing specific antibody for simultaneous detection of both species, as well as for
243 differential detection (14). To date, there is no report on the application of pyruvate
244 phosphate kinase for diagnosis of amoebiasis. Further studies on this protein will be
245 performed, which include production of the recombinant form of the protein and testing
246 with a larger panel of serum samples.

247

248 **ACKNOWLEDGEMENTS**

249 This study was funded by FRGS grant No. 203/CIPPM/6711122, USM-RU Grant
250 No. 1001/PPSK/813009, and USM-RU-PGRS Grant No. 1001/INFORMM/8032030. The
251 first author is a recipient of USM Fellowship, and the third author received financial
252 support from the Vice Chancellor's Award.

253

254 **REFERENCES**

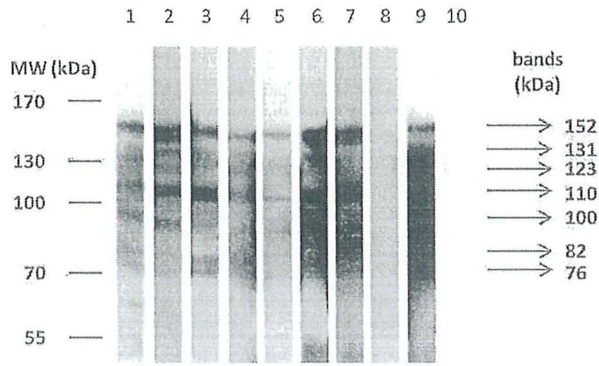
255 1. **Akgun, Y., I.H. Tacyildiz, and Y. Celik.** 1999. Amebic liver abscess: changing
256 trends over 20 years. World J. Surg. **23:**102-106.
257 2. **Diamond, L.S., D.R. Harlow, and C.C. Cunnick.** 1978. A new medium for the
258 axenic cultivation of *Entamoeba histolytica* and other *Entamoeba.* Trans R. Soc.
259 Trop. Med. Hyg. **72:**431-432.
260 3. **Flores, M.S., et al.** 2005. Preparation of *Entamoeba histolytica* antigens without
261 enzymatic inhibitors. Parasitology. **131:**231-236.

262 4. **Fotedar, R., et al.** 2007. Laboratory diagnostic techniques for *Entamoeba* species.
263 Clin. Microbiol. Rev. **20:**511-532.
264 5. **Haque, R., et al.** 1997. *Entamoeba histolytica* and *Entamoeba dispar* infection in
265 children in Bangladesh. The Journal of infectious diseases. **175:**734-736.
266 6. **Haque, R. and W.A. Petri, Jr.** 2006. Diagnosis of amebiasis in Bangladesh.
267 Arch. Med. Res. **37:**273-276.
268 7. **Laemmli, U.K.** 1970. Cleavage of structural proteins during the assembly of the
269 head of bacteriophage T4. Nature **227:**680-685.
270 8. **Pal, S., et al.** 1996. Comparative evaluation of somatic & excretory-secretory
271 antigens of *Entamoeba histolytica* in serodiagnosis of human amoebiasis by
272 ELISA. Indian J. Med. Res. **104:**152-156.
273 9. **Paul, J., S. Srivastava, and S. Bhattacharya.** 2007.Molecular methods for
274 diagnosis of *Entamoeba histolytica* in a clinical setting: an overview. Exp.
275 Parasitol. **116:**35-43.
276 10. **Petri, W.A., Jr. and U. Singh.** 1999.Diagnosis and management of amebiasis.
277 Clin. Infect. Dis. **29:**1117-1125.
278 11. **Que, X. and S.L. Reed.** 2000. Cysteine proteinases and the pathogenesis of
279 amebiasis. Clin. Microbiol. Rev. **13:**196-206.
280 12. **Saavedra-Lira, E., L. Ramirez-Silva, and R. Perez-Montfort.** 1998.
281 Expression and characterization of recombinant pyruvate phosphate dikinase from
282 *Entamoeba histolytica.* Biochim. Biophys. Acta. **1382:**47-54.
283 13. **Sengupta, S., et al.** 2000. Role of excretory-secretory products of *Entamoeba*
284 *histolytica* in human amebiasis. Arch. Med. Res. **31:**226-228.

285 14. **Slamovits, C.H. and P.J. Keeling.** 2006. Pyruvate-phosphate dikinase of
286 oxymonads and parabasalia and the evolution of pyrophosphate-dependent
287 glycolysis in anaerobic eukaryotes. Eukaryot Cell. **5**:148-154.
288 15. **Stephen, P., et al.** 2008. Molecular modeling on pyruvate phosphate dikinase of
289 *Entamoeba histolytica* and in silico virtual screening for novel inhibitors. J.
290 Comput. Aided Mol. Des. **22**:647-660.
291 16. **Tanyuksel, M. and W.A. Petri, Jr.** 2003. Laboratory diagnosis of amebiasis.
292 Clin. Microbiol. Rev. **16**:713-729.
293 17. **Walsh, J.A.** 1986. Problems in recognition and diagnosis of amebiasis: estimation
294 of the global magnitude of morbidity and mortality. Rev. Infect. Dis. **8**:228-238.
295 18. **WHO, WHO/PAHO/UNESCO.** 1997. A consultation with experts on
296 amoebiasis. Mexico City, Mexico 28-29 January. Epidemiol. Bull. **18**:13-14.
297 19. **Wong, W.K., et al.** 2011. Comparison of protein-free defined media, and effect
298 of L: -cysteine and ascorbic acid supplementation on viability of axenic
299 *Entamoeba histolytica.* Parasitol. Res. **108**:425-30.
300 20. **Zambrano-Villa, S. et al.** 2002. How protozoan parasites evade the immune
301 response. Trends in Parasitology. **18**:272-278.
302 21. **Zeehaida, M., et al.** 2008. A study on the usefulness of Techlab *Entamoeba*
303 *histolytica* II antigen detection ELISA in the diagnosis of amoebic liver abscess
304 (ALA) at Hospital Universiti Sains Malaysia (HUSM), Kelantan, Malaysia. Trop.
305 Biomed. **25**:209-216.

306 22. **Zeehaida, M., et al.** 2009. Analysis of indirect hemagglutination assay results
307 among patients with amoebic liver abscess. International Medical Journal.
308 **16:**195-199.
309
310

1
2



3

4 Figure 1 Representative IgG blot of ESA probed with human serum samples. Lanes

5 1-7: individual ALA serum samples from Group A; Lane 8: pooled IHA negative serum

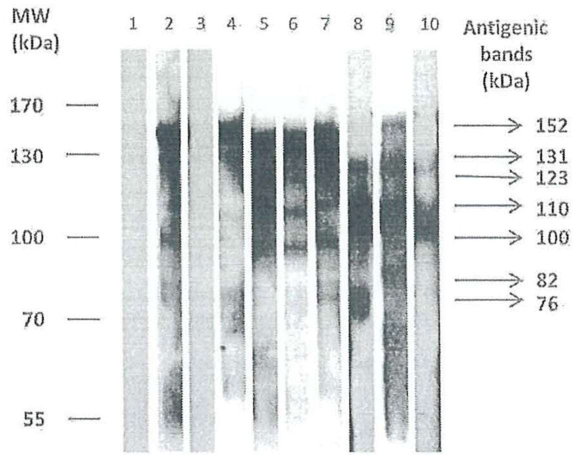
6 sample (Group C); Lane 9: pooled ALA serum sample (positive control); Lane 10: TBS

7

1

2

3



4

5 Figure 2 Representative IgG blot of ESA when probed with human serum samples.

6 Lane 1: TBS; Lane 2: pooled ALA serum sample (positive control); Lane 3: pooled IHA

7 negative serum sample (Group D); Lanes 4-10: individual ALA serum samples from

8 Group B.

Table 1 Sensitivity and specificity of antigenic bands of ESA from IgG blots

Antigenic band, kDa	**Human ALA serum samples, Group A, % [n=24]**	**Human ALA serum samples, Group B, % [n=14]**	**Sensitivity, % Mean (A,B) [n = 38]**	**Specificity, % Mean (C,D) [n = 63]**
152	**79**	**93**	**84**	**100**
131	46	43	45	100
123	17	14	16	100
110	**79**	**86**	**82**	**100**
100	21	14	18	100
82	38	43	39	100
76	38	43	39	100

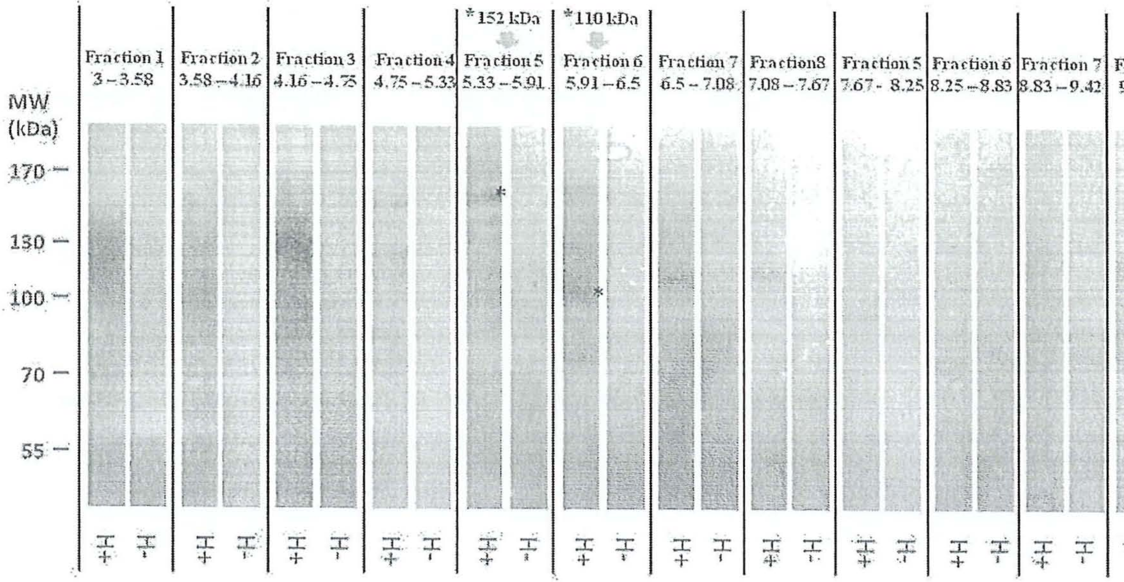
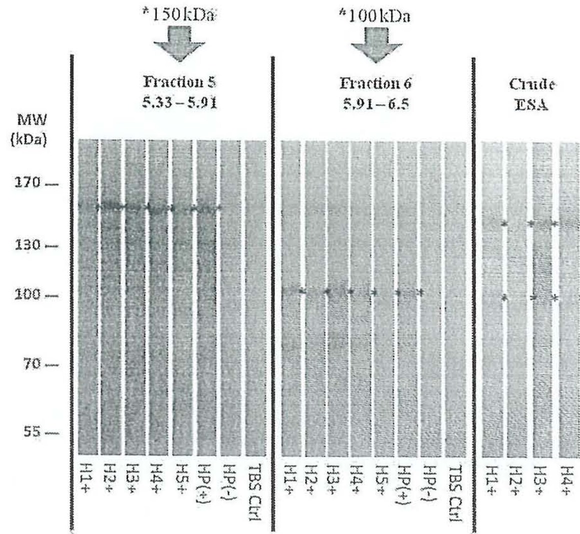


Figure 3 IgG blot of ESA separated by 2-DE, and probed with human serum samples. H+: pooled positive ALA serum sample from Group B; H-: pooled IHA negative serum sample (Group C).

(positive control); HP- : pooled IHA negative serum sample (group D).

1



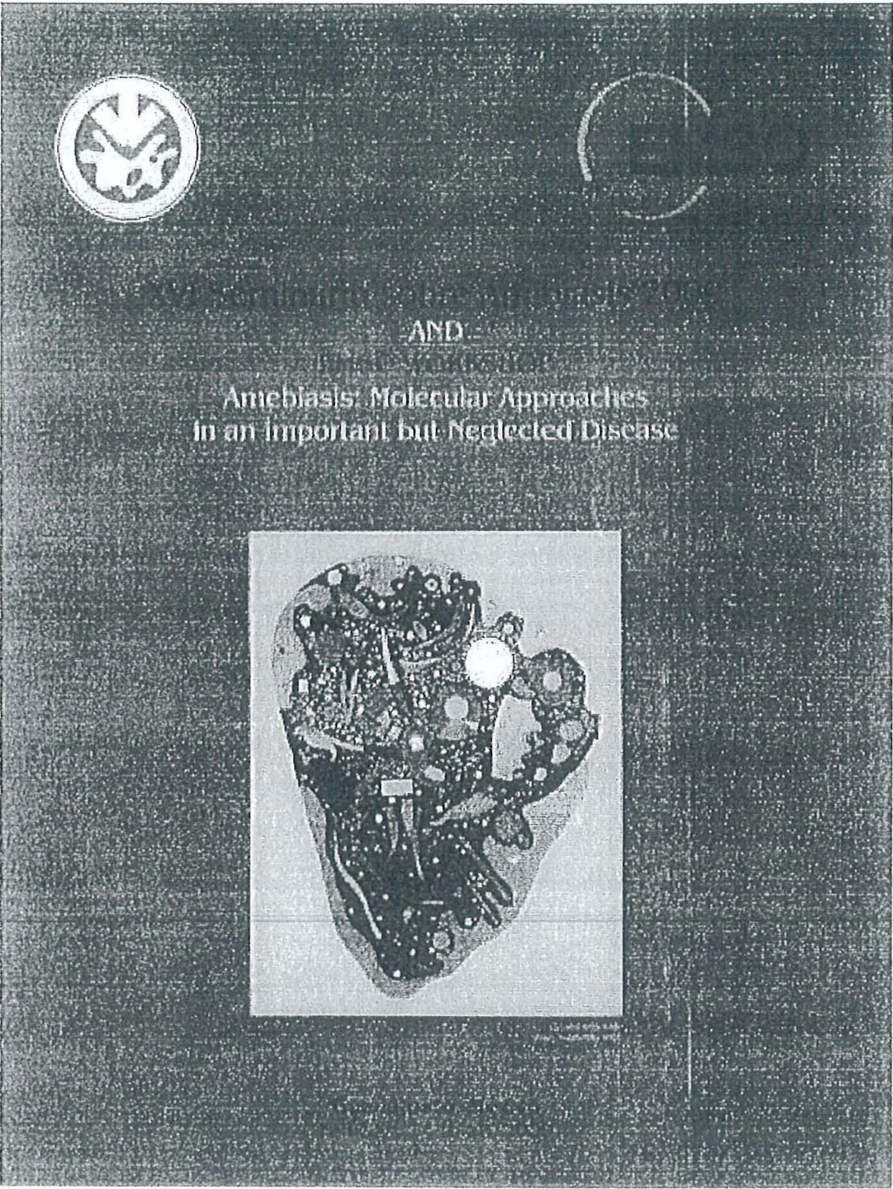
2

3 Figure 4 IgG blot of ESA separated by 2-DE and probed with human serum

4 samples. H1+-H5+: individual ALA serum samples from Group B; HP+: pooled ALA

5 serum sample (positive control); HP- : pooled IHA negative serum sample (group D).

ORAL PRESENTATION:



CHALLENGES IN DIAGNOSIS OF AMOEBIC LIVER ABSCESS: A HOSPITAL UNIVERSITY SAINS MALAYSIA EXPERIENCE

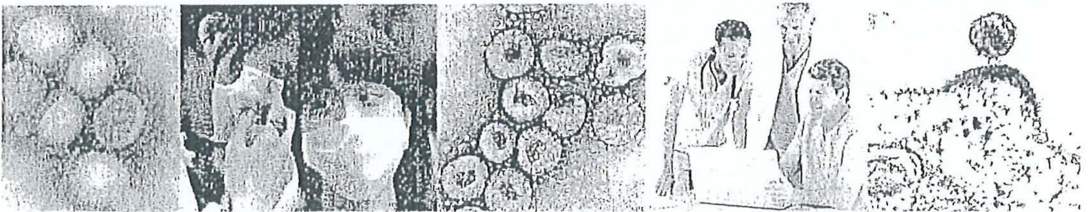
Zeehaida, M[1], Lim B.H[2*], Wan Nor Amilah, W.A.W[2], Amry, A.R[3], Hassan S.[4], Sarimah A[5] and Rahmah, N[6].

[1] Department of Medical Microbiology and Parasitology, [2]Biomedicine Programme, School of Health Sciences, [3]Department of Medicine, [4] Department of Surgery, [5]Biostatistics Unit, School of Medical Sciences, University Sains Malaysia, 16150 Kubang Kerian, Kelantan,[6] Institute for Research in Molecular Medicine, University Sains Malaysia .

Amoebic liver abscess (ALA) is the most common extraintestinal manifestation of amoebiasis in South East Asian countries and Mexico. Diagnosis is based on clinical symptoms and signs; occurrence of space occupying lesion in liver detected by imaging techniques, positive amoebic serology and clinical response to antiamoebic therapy with metronidazole. The amoebic serological assay employed at Hospital University Sains Malaysia (HUSM), Kelantan is the commercial indirect haemagglutination assay (IHA) kit which detects anti-*Entamoeba histolytica* antibodies in serum samples. During the study period from January 2005 till June 2006, there were 43 clinical or suspected cases of ALA. On admission, all patients presented with fever. Thirty nine of them had abdominal pain; 9 had history of passing loose stools; 42 presented with hepatomegaly while 15 had jaundice; and 37 presented with leucocytosis. Thirty three patients had liver abscesses in their right lobe, 6 with abscesses in the left lobe and 4 had abscesses in both lobes of the liver. The IHA test were positive (titer more than or equal to 1:256) in 33 (76.7%) patients. Forty (wo of the patients were treated with intravenous metronidazole 500 mg every 8 hours and continued with oral metronidazole when the patients were discharged. In the amoebiasis endemic setting in Kelantan, interpretation of IHA results can be problematic due to the high background antibody levels Therefore, a simple, rapid, noninvasive test with high sensitivity and specificity for laboratory diagnosis of ALA is urgently needed..

3rd NATIONAL CONFERENCE ON INFECTIOUS DISEASES

Innovative Approaches in Infectious Diseases: Towards Sustainable Healthcare



26-27 October 2009
Renaissance Kota Bharu Hotel

Department of Medical Microbiology & Parasitology
School of Medical Sciences
Universiti Sains Malaysia

designed by hairy_116@hotmail.com /0139995894

POSTER PRESENTATION

P1: RAPID STAINING OF *Entamoeba histolytica* TROPHOZOITES IN FRESH STOOL SAMPLE

Tan Zi Ning[1], Wong Weng Kin[3], Nik Zairi Zakaria[2], Abdullah Bujang[2], Rahmah Noordin[3], Zeehaida Mohamed[2], Pattabhiraman Lalitha[4], Tan Gim Cheong[2]. Lim Boon Huat[1]

[1]School of Health Sciences, Universiti Sains Malaysia
[2]Department of Medical Microbiology and Parasitology.School of Medical Sciences, Universiti Sains Malaysia
[3]Institute for Research in Molecular Medicine, Universiti Sains Malaysia
[4]Faculty of Applied Sciences, AIMST University, Malaysia

Background and aims

Amoebiasis is a ubiquitous disease caused by *Entamoeba histolytica*, an intestinal parasitic protozoon. Commercially available *E. histolytica* antigen detection kits for detection of trophozoites in stool samples are not cost effective for use in most of the underdeveloped endemic countries. Thus, microscopy technique is still relevant for detection of intestinal amoebiasis However, a major setback in the latter method is the requirement for freshly collected faecal samples. Furthermore, there are no reports on the survival time of trophozoites in fresh faecal sample Numerous staining techniques are employed for the detection of trophozoites and/or cysts, and Wheatley trichrome staining is the routine technique employed at the Department of Medical Microbiology and Parasitology, School of Medical Sciences, USM The objective of this study is to determine the period of viability of trophozoites in spiked stool sample Secondly, the efficacy of Wheatley trichrome technique was compared with the Eosin and Periodic Acid Schiff (PAS) techniques.

Methods

Two grams of fresh faecal sample were spiked with $1x10^6$ trophozoites and about 2 mg duplicates of the spiked sample were stained with Trypan blue, and the viability determined under the microscope using a Neubauer chamber. The process was repeated at 30 minute intervals for 8 hours. The efficacies of the three types of stains were compared based on the ease of detection of the characteristic features of the trophozoites.

Results

The results showed that the trophozoites were still viable within 8 hours but highly decreased in number; and the Eosin staining technique was the easiest to perform.

Conclusion

Eosin staining technique is recommended for detection of trophozoites in stool sample collected within 8 hours.

PROCEEDINGS

INFORMM Students' Colloquium 2009

PUBLISH OR PERISH

PHARMACOGENOMICS AND GENOMICS
PARASITOLOGY
TYPHOID AND ENTERIC DISEASES
CANCER/ NATURAL PRODUCT
IMMUNOLOGY
MOLECULAR BIOLOGY

INFORMM, Health Campus, USM
10-11 October 2009
Organized by INFORMM Students

Comparison of Growth Media and Effect of Cysteine and Ascorbic acid Supplementation on *in-vitro* Culture of *Entamoeba histolytica*

Wong Weng Kin[1], Tan Zi Ning[2], Lim Boon Huat[2], Zeehaida Mohamed[3], Alfonso Olivos Garcia[4], Rahmah Noordin[1]

[1]*Institute for Research in Molecular Medicine, Universiti Sains Malaysia*

[2]*School of Health Sciences, Universiti Sains Malaysia*

[3]*Department of Medical Microbiology and Parasitology, School of Medical Sciences, Universiti Sains Malaysia,*

[4]*Department of Experimental Medicine, Medical School, Universidad Nacional Autónoma de México*

Entamoeba histolytica is the etiologic agent for amoebiasis. The excretory-secretory (ES) products of this parasite have been shown to contain virulence factors that contribute to its invasive characteristics. Besides, ES products also possess antigenic properties that are useful for diagnostic application. Therefore, a protein-free medium that can sustain the viability of *E. histolytica* for longer duration will greatly assist in the study on these ES products. In the present study, we compared four different protein-free media in maintaining 95% viability of the parasite namely phosphate buffered saline for amoeba (PBS-A), Hank's balance salt solution (HBSS), Roswell Memorial Park Institute medium, No 1640 (RPMI-1640) and Dulbecco's Modified Eagle Medium (DMEM). Concurrently we determined the effect of adding cysteine and ascorbic acid to the medium on the viability of the organism. With addition of the supplement, viability of *E. histolytica* at 95% was prolonged for up to 6 hours, equivalent to a 1.5 fold hour increment in DMEM; 5 hours (2.5 folds) in RPMI-1640 and 2 hours (no increment) in PBS-A and HBSS. In conclusion, DMEM was found to be the best protein-free medium in maintaining viability of *E. histolytica in-vitro,* and that DMEM and RPMI media with added cysteine and ascorbic acid sustained the parasites' viability longer than without the supplement.

Keywords: *Entamoeba histolytica*, Protein free media, Excretory secretory (ES) products, Cysteine and ascorbic acid

Organized by:

ABSTRACT BOOK

INTERNATIONAL SCIENTIFIC CONFERENCE

Recent Trends & Future Perspectives Towards the Protection of Health

6 – 8 November 200
First World Hot
Genting Highlands, Malays

Support Provided by:

Malaysian Nurses Association

Ministry of Health Malaysia

Cysteine and Ascorbic Acid Supplement Prolonged Viability of *Entamoeba histolytica* in Pro Free Media

Wong Weng Kin[1], Tan Zi Ning[2], Lim Boon Huat[2], Zeehaida Mohamed[3], Alfonso Olivos Garcia[4], Rahmah Noordin[1]
[1]*Institute for Research in Molecular Medicine, Universiti Sains Malaysia, Malaysia*
[2]*School of Health Sciences, Universiti Sains Malaysia, Malaysia*
[3]*Department of Medical Microbiology and Parasitology, School of Medical Sciences, Universiti Sains Malaysia, Malaysia*
[4]*Department of Experimental Medicine, Medical School, Universidad Nacional Autónoma de México, Mexico*

Entamoeba histolytica is the etiologic agent for amoebiasis. The excretory-secretory (ES) products of this parasite co virulence factors that contribute to its invasive characteristic. ES products also possess antigenic properties that are u for diagnostic application. Thus, a protein-free medium that can sustain the viability of *E. histolytica* for longer duratior facilitate in the study on these ES products. In the present study, we investigated the effect of adding cysteine and asco acid in maintaining the viability of *E. histolytica* in several common protein-free media namely phosphate buffer saline amoeba (PBS-A), Hank's balance salt solution (HBSS), Roswell Memorial Park Institute medium, No 1640 (RPMI-1640) Dulbecco's Modified Eagle Medium (DMEM). The target of achieving 95% viability of *E. histolytica* was determined using Tr blue exclusion method. The results showed that the viability of *E. histolytica* was prolonged in all media supplemented cysteine/ascorbic acid. With these supplements, 95 % viability of *E. histolytica* was prolonged for up to 5 hours, equivalen a 2.5 fold hour increment in RPMI-1640 and 6 hours (1.5 folds) in DMEM, whereas, in PBS-A and HBSS the viabilities *histolytica* were prolonged for only 2 hours. In conclusion, cysteine and ascorbic acid were found to significantly prolong viability of *E. histolytica* in DMEM protein-free medium. This enables the use of this medium for production of *E. histolytic* products, thus reducing the interference of unwanted proteins present in serum supplemented medium.

PHPP40

Strategic Evaluation of Factors Involved in the Development of Nutraceuticals

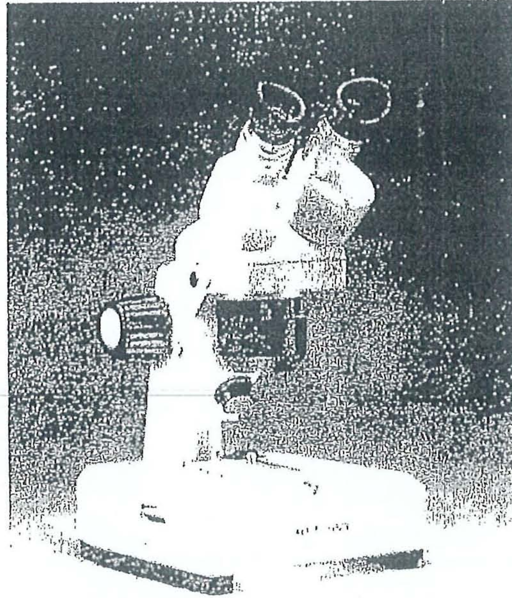
Shilpa Dua, Ajay Pise, N Udupa
Department of Pharmacy Management, Manipal College of Pharmaceutical Sciences, Manipal-576 104, Karnataka, India

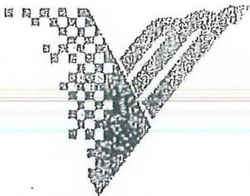
This study aims to analyse the factors responsible for the growth of nutraceuticals, to find out hurdles in the development nutraceuticals, to find out factors responsible for the development of nutraceuticals, and to propose recommendations f the development of nutraceuticals Secondary data collection method was adopted for the study, Data was collected fro authentic sources including books, official websites, journals, news paper articles and related magazines, Collected data w converted in to information to derive the results, Results and conclusion of this study were derived after analysing the da During this study we have observed following hurdles in the development of Nutraceuticals- Dilemma about the conce of nutraceuticals, absence of clear cut distinction between nutraceuticals, dietary supplement, and functional foods, harmonized definition of nutraceuticals, Nutraceutical concept is used as marketing gimmick, lack of adequate research the area of nutraceuticals, absence of regulatory guidelines in many countries is undue advantage for healthcare compani lack of proper categorization of nutraceuticals. Following advantages and potentials were observed for nutraceutica Increasing purchasing power and per capita income will help to boost the demand for nutraceuticals, increasing awarene about health maintenance and increasing cost of drugs, fear of disease and disease conditions, Government initiativ for public health maintenance, baby boomers are contributing more in increasing the demand of nutraceuticals. Fro above study we can conclude that there is a need of a scientific and horminised definition, need of clearly differentiati nutraceuticals from dietary supplements, and functional foods, need of properly framed hormonised regulations f manufacturing, advertising and selling of nutraceuticals, more encouragement should be given to the research in the ar of nutraceuticals. Nutraceuticals should be classified scientifically in acceptable format.

Makmal Veterinar Kawasan Kota Bharu

MESYUARAT KUMPULAN PAKAR PARASITOLOGI DAN HEAMATOLOGI

BIL 1/2010

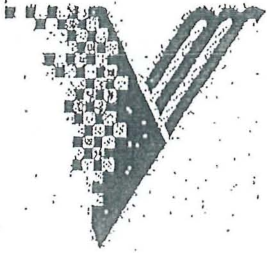




Makmal-makmal Veterinar Kawasan

Jabatan Perkhidmatan Veterinar

09-7654339 MVK KOTA BHARU KEL 563 P01 APR 01 '10 10:



REGIONAL VETERINARY LABORATORY
MAKMAL VETERINAR KAWASAN KOTA BHARU
Kompleks Jabatan Perkhidmatan Haiwan
16150 Kubang Kerian
Kota Bharu
Kelantan
Malaysia

Tarikh:

No. Talipon: 609-7652815	No. Fax: 609-7654339
Daripada: MVK KB (Dr Lee)	Alamat: USM
Kepada: USM (Dr Lim Boon Hock)	No. Fax: 09-7677515
Bil. Muka Surat: 5	Termasuk Muka Surat Ini:- 6

Catatan:-

Thank you for responding to our meeting / invitation. Beside, we like to know what do USM have for Parasitologi and Heamatology. Hope we can co-operate together to share the knowledge.
Thanks again.

Oral Presentation by Lim BH, Zeehaida, M. Nik Zairi, Z & Rahmah, N. Diagnosis of Parasitic Diseases in Hospital Universiti Sains Malaysia

Tandatangan Penghantar:..

15th National Conference on

MEDICAL AND HEALTH SCIENCES 2010

Caring For People With

Special Needs

21 & 22 July 2010

Organized by:

School of Health Sciences, Universiti Sains Malaysia

DETECTION OF INVASIVE AMOEBIASIS BY ELISA USING DIFFERENT ANTIGEN PREPARATION METHODS

Tan Z.N.[1], Wong W.K.[2], Zeehaida M.[3], Rahmah N.[2] & Lim B. H.[1]

[1] School of Health Sciences, [2]Institute for Research in Molecular Medicine, [3]Department of Medical Microbiology and Parasitology School of Medical Sciences, Health Campus, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia

Amoebiasis is an invasive disease caused by a well-known enteric protozoan, *Entamoeba histolytica*. It is a ubiquitous disease and 100 000 people are estimated to die annually. Numerous diagnostic tests have been performed to diagnose this illness; however, serodiagnosis has become a preferable method in most of the diagnostic laboratories. In Hospital Universiti Sains Malaysia (HUSM), Kelantan, indirect hemeagglutination assay (IHA), Cellognost ® Amoebiasis (Dade Behring Marburg GmbH, Germany) is being employed to diagnose invasive amoebiasis based on detection of specific antibodies to *E. histolytica* in patients' serum samples. Although this commercial IHA kit has been reported as a very sensitive diagnostic tool, it is not cost effective to be used in mass screening of invasive amoebiasis, particularly in poor endemic countries. The aim of this study is to compare the sensitivity and specificity of two different antigen preparations, namely crude soluble antigen (CSA) and ether extract antigen (EEA) in an enzyme-linked immunosorbent assay (ELISA) format. Here, 16 IHA seropositive samples and 16 seronegative samples were used to compare the sensitivity and specificity of the two different antigen preparations. Prior to that, parameters such as concentrations of antigens used for each well, dilutions of primary and secondary antibodies, incubation time and washing steps were optimized and subsequently the respective cut-off values (COV) were determined. The results showed that the sensitivity and specificity of CSA in detection of invasive amoebiasis were 93.75% (15/16) and 75% (12/26) respectively. In comparison, the EEA results indicated sensitivity of 81.25% (13/16) and specificity of 93.75% (15/16). Both types of antigen preparations revealed high sensitivity of above 80%, but EEA showed higher specificity as compared to CSA. In conclusion, EEA is recommended to as an in-house ELISA for diagnosis of invasive amoebiasis at HUSM.

15th National Conference on

MEDICAL AND HEALTH SCIENCES 2010

USM UNIVERSITI SAINS MALAYSIA

Caring For People With

Special Needs

21 & 22 July 2010

Organized by:

School of Health Sciences, Universiti Sains Malaysia

OM04

IDENTIFICATION OF POTENTIAL EXCRETORY SECRETORY ANTIGENS FOR DIAGNOSIS OF AMOEBIC LIVER ABSCESS

Wong, W. K.[1], Tan, Z. N.[2], Lim, B.H.[2], Zeehaida, M.[3] & Rahmah, N.[1]

[1]Institute for Research in Molecular Medicine, [2]School of Health Sciences, Universiti Sains Malaysia [3]Department of Medical Microbiology and Parasitology, School of Medical Sciences, Health Campus, Universiti Sains Malaysia 16150 Kubang Kerian, Kelantan, Malaysia

Entamoeba histolytica is the etiologic agent for amoebiasis, which affects an estimated 10 % of the world population. Diagnosis of extraintestinal amoebiasis, such as amoebic liver abscess (ALA) is often based on clinical manifestations, radiology imaging and serological test. ALA is potentially fatal if early diagnosis followed by appropriate treatment is not sought. Indirect Haemagglutinin assay (IHA) is a common antibody detection test for diagnosis of ALA, but various reports revealed that its diagnostic sensitivity is low. Previous studies had shown that excretory secretory antigen (ESA) of *E. histolytica* was antigenic and possessed potential diagnostic value. Therefore, the presence work is aimed at identifying potential antigen(s) to improve the sensitivity of serodiagnosis for ALA. ESA was produced from DMEM culture medium supplemented with cysteine and ascorbic acid that supported >95% viable *E. histolytica* trophozoites. Subsequently, the antigenic profile of ESA was analysed using Western blot probed with sera from ALA patients (n = 7), with positive IHA results and whose pus samples were positive for *E. histolytica* by realtime PCR.. Four antigenic bands were identified as potential candidates for further studies namely 185 kDa (5/7), 106 kDa (7/7), 96 kDa (7/7), and 40 kDa (6/7).

36th Annual Conference of The Malaysian Society for Biochemistry & Molecular Biology (MSBMB)

27th – 28th July 2011
Eastin Hotel,
Petaling Jaya, Selangor

Oral 11

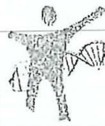
BIOCHEMICAL CHARACTERIZATION OF *Entamoeba histolytica* CHOLINE KINASE

CHANG CHIAT HAN, FEW LING LING, LIM BOON HUAT AND SEE TOO WEI CUN

School of Health Sciences, Universiti Sains Malaysia, Health Campus, 16150 Kubang Kerian, Kelantan Darul Naim, Malaysia.

Entamoeba histolytica is one of the most widespread and clinically important protozoa parasites. Annually, there are more than 50 million cases of amoebiasis being reported with about 100,000 deaths. Patients may develop amoebic dysentery, liver or brain abscesses which are fatal if untreated. Fractionation of *E. histolytica* lipid showed that its total phospholipid was predominated by phosphatidylcholine (PtdCho). The knowledge of PtdCho biosynthesis is pertinent to understand *E. histolytica* lipid biochemistry and their physiological roles as well as its possible relationship to infection. The objective of this study is to determine the biochemical properties of putative *E. histolytica* choline kinase (EhCK), the first enzyme participates in the main biosynthesis pathway of PtdCho. EhCK activity was measured by spectrophotometric PK-LDH coupled assay with choline or ethanolamine as substrate. This assay system was used to determine the optimum pH, temperature, kinetic parameters and effects of different divalent metal ions on enzyme activity. Our result showed that EhCK was active at alkaline pH, with the optimum activity at pH 8.0. The enzyme possessed the highest activity at about 40°C, and became unstable at higher temperature. EhCK showed V_{max} and K_m against choline at 0.955 μmol/min/mg and 235 μM, respectively. We also discovered that EhCK preferred manganese ion over magnesium ion as its cofactor. As a conclusion, we proved that EhCK was active and reported the kinetic parameters of this enzyme. Its metal ion cofactor preference is a unique feature as compared to choline kinases reported in other species.

amdi
Advanced Medical and Dental Institute



1st AMDI INTERNATIONAL BIOHEALTH SCIENCE CONFERENCE (IBSC) 2010

Infectious Diseases - Current Challenges
Programme And Abstracts Book

29th November - 1st December 2010
Bayview Beach Resort
Penang, Malaysia.

CID019.P

Staining of *Entamoeba histolytica* in hamster liver

Tan Zi Ning[1], Wong Weng Kin[2], Shyamoli Mustafa[1], Arefuddin Ahmed[1], Rahmah Noordin[2], See Too Wei Cun[1], Tan Gim Cheong[3], Alfonso Olivos-Garcia[4], Lim Boon Huat[1]*

[1]School of Health Sciences, Universiti Sains Malaysia, Kubang Kerian, Kelantan, Malaysia, [2]Institute for Research in Molecular Medicine, Universiti Sains Malaysia, Penang, Malaysia, [3]Department of Medical Microbiology and Parasitology, School of Medical Sciences, Universiti Sains Malaysia, Kubang Kerian, Kelantan, Malaysia, [4]Departamento de Medicina Experimental, Facultad de Medicina, Universidad Nacional Autónoma de México, México D.F., México

*Corresponding Author: Tan Zi Ning
E-mail: zining0316@gmail.com

Background/Aim: Amoebic liver abscess (ALA) is the most common clinical presentation of extraintestinal infection with *Entamoeba histolytica*. This disease leads to significant morbidity and mortality in endemic countries. Although less than 1% of patients infected with *E. histolytica* develop ALA, this still represents a large number of patients throughout the world. Molecular-based methods have been used for diagnosis of ALA; however most of them are not suitable for resource-limited developing countries. Thus, the conventional staining technique is still relevant in these laboratories. The objective of this study is to compare the efficacy of three different stains namely haematoxylin and eosin (H&E), periodic-acid Schiff (PAS) and immunohistochemical (IHC) stains for detection of *E. histolytica* trophozoites in liver tissue of hamster with ALA.

Methodology: ALA was experimentally induced in hamster by injecting HM-1: IMSS *E. histolytica* trophozoites through the portal vein. Liver tissue samples were aseptically removed and processed for histology. The processed tissues were stained separately with H&E and PAS stains. IHC stain was performed on the processed tissues by using polyclonal antibody against amoebic trophozoite.

Results: Tissue necrosis and amoebic trophozoites were observed in all the three kinds of stained tissue sections. However, the use of IHC stain resulted in a more distinct appearance of the trophozoites in the infected liver tissues as compared to the other two staining techniques.

Conclusions: IHC staining was found to be the best method to identify *E. histolytica* trophozoites in tissue with ALA. Similarly it is expected to be a very good stain for identifying the trophozoites in patient's liver abscess aspirates. Therefore its use can be recommended for the diagnosis of ALA.

1st AMDI INTERNATIONAL BIOHEALTH SCIENCE CONFERENCE (IBSC) 2010

Infectious Diseases - Current Challenges
Programme And Abstracts Book

29th November - 1st December 2010
Bayview Beach Resort
Penang, Malaysia.

MB008.O

Cloning, expression and characterization of *Entamoeba histolytica* choline/ethanolamine kinase

Chang Chiat Han[*], *Tan Zi Ning, Few Ling Ling, Lim Boon Huat, See Too Wei Cun*

School of Health Sciences, Universiti Sains Malaysia, Kubang Kerian, Kelantan, Malaysia

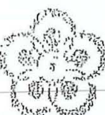
*Corresponding Author: Chang Chiat Han
E-mail: chiathan@gmail.com

Background/Aim: *Entamoeba histolytica* is one of the most widespread protozoa which causes amoebic liver abscess and intestinal disease. It is the second most death-causing parasite after *Plasmodium falciparum* with 70,000 deaths annually. *E. histolytica* is one of the primitive eukaryote known. Its total lipid is predominated by phospholipid (60-70%) consisting of phosphatidylethanolamine (PtdEtn) and phosphatidylcholine (PtdCho). Phospholipid has been reported to protect *E. histolytica* from self-toxin. Hence, the understanding of PtdCho and PtdEtn biosynthesis is pertinent to control its infection. This study aims to clone, express, purify and characterize *E. histolytica* putative choline/ethanolamine kinase (EhCK/EK), the first enzyme in the main biosynthetic pathway of PtdCho and PtdEtn.

Methodology: Using human choline kinase α1 amino acid sequence as a query, *E. histolytica* putative choline/ethanolamine kinases were identified using NCBI blastp program. Gene candidate was amplified from *E. histolytica* genomic DNA by polymerase chain reaction and inserted into pGEX-RB vector to be expressed as Glutathione S-transferase (GST) fusion EhCK/EK1. The construct was then transformed into *Escherichia coli* BL-21 strain for protein expression. The activity of the purified EhCK/EK1 was shown by spectrophotometric pyruvate kinase-lactate dehydrogenase coupled assay. The mRNA expression of EhCK/EK1 in *E. histolytica* was detected by RT-PCR.

Results: EhCK/EK1 gene was amplified and expressed as a recombinant GST fusion protein. The protein was purified to homogeneity. It was shown to possess activity with choline but not ethanolamine as substrate. RT-PCR result showed that EhCK/EK1 gene was expressed in *E. histolytica*.

Conclusions: We reported the first isolation and characterization of a CK/EK from *E. histolytica*. The enzyme was shown to be a choline-specific kinase and confirmed to be expressed by *E. histolytica*. This enzyme is currently being further studied to characterize its biochemical properties.



東京医科歯科大学
TOKYO MEDICAL AND DENTAL UNIVERSITY

International Summer Program 201

5-8 September 2010

Infection and Immunity

PROGRAM & ABSTRACT BOOK

Tokyo Medical and Dental University http://www.tmd.ac.jp/
International Summer Program 2010 http://www.tmd.ac.jp/TMDU-e/isc/isp2010/index.html

Poster No. 13

Weng Kin Wong

(Universiti Sains Malaysia)

Title: Demonstration of potential antigenic components of excretory-secretory antigen for serodiagnosis of amoebic liver abscess

Abstract

Wong Weng Kin[1], Tan Zi Ning[2], Nurulhasanah Othman[1], Lim Boon Huat[2], Zeehaida Mohamed[3] and Rahmah Noordin[1]

[1]Institute for Research in Molecular Medicine, Universiti Sains Malaysia, Malaysia

[2]School of Health Sciences, Universiti Sains Malaysia, Malaysia

[3]Department of Medical Microbiology and Parasitology, School of Medical Sciences, Universiti Sains Malaysia, Malaysia

Amoebiasis is a cosmopolitan parasitic disease caused by *Entamoeba histolytica*. The disease has become a globalized problem due to the ease of world travel. According to WHO report, this disease affects about 10% of the world population. Humans get infected through the ingestion of the infective stage cysts. The symptomatic patient may present with amoebic dysentery, amoebic colitis, and amoebic liver abscess (ALA). ALA is potentially fatal if early diagnosis and treatment is not sought. Currently, the diagnosis for ALA often depends on clinical symptoms, radiological imaging and serological test. Indirect Haemagglutinin (IHA) and TechLab E. histolytica II are commonly used for the diagnosis in Amoebiasis. However, previous reports revealed that these two tests showed low sensitivity for the serodiagnosis of ALA.

The present research aims at identifying novel potential antigen(s) of *E. histolytica* that can improve the serodiagnosis of ALA. We are investigating the excretory-secretory antigens (ESA) of this parasite as reports had shown that ESA showed good sensitivity for detection of amoebic cysts passers, amoebic dysentery cases and ALA. Seven serum samples from human cases of ALA were used, these were positive by IHA as well by real-time PCR of the abscess fluid. Thus far, our Western blot study had shown that ESA (7/7) showed better sensitivity for detection of ALA as compared to crude soluble antigen (5/7).

Acknowledgement

This study was supported by grant USM-RU-PGRS Grant i.e. No. 1001/INFORMM/8032030, USM-RU Grant i.e. No. 1001/PPSK/813009, and FRGS grant i.e. No. 203/CIPPM/6711122. The author thanks for the support of USM Fellowship.

PROGRAM AND ABSTRACT BOOK

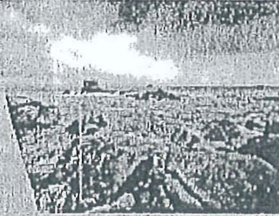
47th Annual Conference of the Malaysian Society of Parasitology & Tropical Medicine

3 & 4 March 2011
IMU Bukit Jalil, Kuala Lumpur Malaysia

Climate Change and its Impact on Public Health

Officiated by
Tan Sri Dato' Dr Abu Bakar Suleiman
President
International Medical University, Kuala Lumpur

Jointly Organised By



OP11

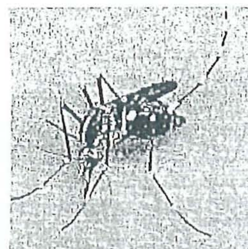
RECOGNITION OF POTENTIAL ANTIGENIC PROTEINS FOR DIAGNOSIS OF AMOEBIC LIVER ABSCESS USING TWO DIFFERENT ANTIGEN PREPARATIONS

Tan Zi Ning[1], Wong Weng Kin[1], Rahmah Noordin[2] and Lim Boon Huat[1]

[1]*School of Health Sciences, Universiti Sains Malaysia, Malaysia*
[2]*Institute for Research in Molecular Medicine, Universiti Sains Malaysia, Malaysia*

Amoebic liver abscess (ALA) is the most common clinical manifestations of amoebiasis in human caused by an enteric protozoan, *Entamoeba histolytica*. This neglected disease has claimed about 100,000 lives and inflicted many more annually. Indirect hemeagglutination assay (IHA), Cellognost ® Amoebiasis (Dade Behring Marburg GmbH, Germany) is frequently used in diagnosis of ALA, but it is costly and not suitable to be used in mass screening of invasive amoebiasis, particularly in endemic developing nations. Thus, the objective of this study is to identify potential antigenic proteins from *E. histolytica* crude soluble antigen (CSA) and ether extract antigens (EEA) for the diagnosis of ALA by using experimentally infected hamster serum samples. Thirty Golden Syrian hamsters were each inoculated with 1×10^6 of *E. histolytica* trophozoites to produce ALA. Each animal was sacrificed with 3x overdose of pentobarbital and the cardiac-punctured blood sample was collected to obtain the serum. The CSA was prepared via sonication of trophozoites while the EEA was prepared by solubilizing the trophozoites using ether. Protein concentrations from both antigen preparations were then determined using Bio-Rad Assay. Subsequently, Western blot analysis on both CSA and EEA based on the hamster ALA serum samples revealed that the ~70kDa as a potentially important antigen for diagnosis of hamster ALA. In conclusion, this antigenic protein set the stage for further study in the diagnosis of human ALA.

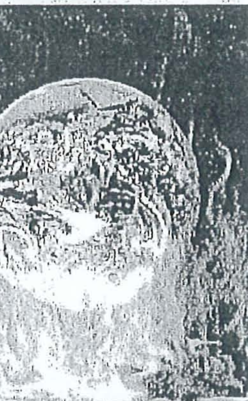
PROGRAM AND ABSTRACT BOOK

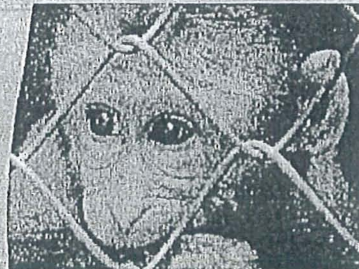


47th Annual Conference of the Malaysian Society of Parasitology & Tropical Medicine

3 & 4 March 2011
IMU Bukit Jalil, Kuala Lumpur Malaysia

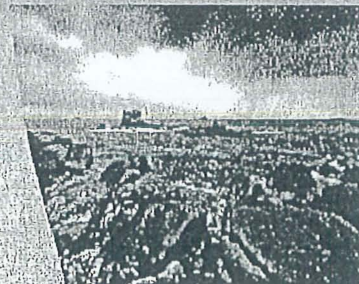
Climate Change and its Impact on Public Health





Officiated by
Tan Sri Dato' Dr Abu Bakar Suleiman
President
International Medical University, Kuala Lumpur

Jointly Organised By



PP32

COMPARISON OF *ENTAMOEBA HISTOLYTICA* PROTEIN PROFILES FROM TWO DIFFERENT PREPARATIONS

Lim Boon Huat[1], Tan Zi Ning[1], Wong Weng Kin[2], Foo Phiaw Chong[1] and Rahmah Noordin[2]

[1]*School of Health Sciences, Universiti Sains Malaysia, Malaysia*
[2]*Institute for Research in Molecular Medicine, Universiti Sains Malaysia, Malaysia*

Entamoeba histolytica is an enteric protozoan that causes intestinal amoebiasis, which may manifests into the fatal amoebic liver abscess (ALA) if left untreated. Previous studies reported that membrane-bound proteins of the parasite could be antigenic and play important role in diagnosis. However, some other amoeba species might share similar membrane-bound proteins. Thus, to increase the specificity of diagnosis, membrane-bound proteins can be removed by ether. The aim of this study is to compare the protein profiles of crude soluble antigen (CSA) and ether extract antigen (EEA) of *E. histolytica*. Firstly, 10×10^6 trophozoites were used to prepare either CSA or EEA. CSA was done by sonication technique while EEA was prepared by adding ether to 1X PBS suspended trophozoites and the supernatant was collected after removing the organic phase. Protein concentrations were determined and both CSA and EEA protein profiles were compared by running 12% SDS-PAGE. Several protein bands such as ~40kDa and ~32kDa not found in EEA protein profile were probably located in the plasma membrane. It is interesting to note that both the antigen preparation techniques showed a few prominent protein bands such as ~100kDa, ~45kDa and ~38kDa. Future study will focus on the antigenicities of these proteins.

PP33

PREVALENCE OF TOXOPLASMA GONDII ANTIBODIES IN WOMEN IN TRIPOLI - LIBYA

El-Gomati KM[1], El Naas AS[1], Rashed AM[1] and Elsaid MMA[2]

[1]*Department of Parasitology, Faculty of Veterinary Medicine, Alfateh University, P.O. Box: 13663*
[2]*Department of Parasitolgy, Faculty of Medical technology, Alfateh University, P.O. Box: 13663*

This study carried out to determine the sero-prevalence of *Toxoplasma gondii* antibodies in women. A total of 240 samples of sera form the Obstetric gyna out patient clinic. The sero-positive were 131 out of 240 women (54.58 %). Among the age group 19-28 year the sero-positive 55 (48.25%) out of 114, comparing to the age group 29-38 year and age group 39-48 year were 62 (60.19%) out 103, 14 (60.87%) out of 23 respectively, but statisticaaly no significance according to age. Also found that the women whom are seropositive and had no history of contact with cats were(54.58%), while women whom are seropositive and have history of contact with cats were (57.4%), statistical no significance difference between seropositive, factor contact with cat, and no difference according their residence at different part of Tripoli-Libya.

Malaysian Symposium

on

Biomedical Science '11

12th-13th of March 2011

Kulliyyah of Science, IIUM Kuantan Campus

"share the knowledge, enlighten the future"

Potentially important antigen of *Entamoeba histolytica* for diagnosis of amoebic liver abscess: An animal model

Lim Boon Huat[1], Aziah Ismail[2], **Siti Shafiqah Anaqi Azham**[1]
School of Health Sciences[1]*, Institute for Molecular medicine*[2]*,*
University Sains Malaysia

Entamoeba histolytica is a causative agent of amoebiasis. Most of the infections are asymptomatic while others range from dysentery to amoebic liver abscess (ALA), which is potentially fatal. The commercially available indirect haemagglutination assay (IHA) is the common detection method for ALA. The assay is expensive, thus is not suitable for use in poor endemic countries. This study aimed to identify potentially important antigen(s) of *E. histolytica* based on the ALA-positive serum samples of Golden Syrian hamsters. The crude soluble antigen (CSA) of *E. histolytica* was prepared from axenic culture of the amoeba strain. After that, SDS-PAGE protein profiling was performed, followed by Western blot analysis. Thirty-two infected hamster serum samples and 7 healthy hamster serum samples were used as the primary antibody, and Tris-buffered saline (TBS) was used as the negative control. Anti-hamster antibody was used as the secondary antibody and tetramethylbenzene (TMB) was used as the detection reagent. The molecular weight of antigenic protein bands were analyzed by using gel pro analyzer. The results showed several antigenic bands with different molecular weights ranging from 70 kDa to 130 kDa. The 97 kDa protein band showed a sensitivity and specificity of 91% and 100% respectively. In conclusion, the protein band was found to be potentially important for detection of ALA in human.

Keywords: Entamoeba histolytica, Diagnosis and Amoebic liver abscess.

Genetic Polymorphisms Of The Inflammatory Bowel Disease 5 (*IBD5*) And Interleukin-23 Receptor (*IL23R*) Genes In Malaysian Crohn's Disease Patients

Chua Kek Heng, Lian Lay Hoong, **Sathya Narayanan A/L Patmanathan**
Department of Molecular Medicine,
University of Malaya

There are strong evidences that support the role of genetic factors in the susceptibility of individuals to inflammatory bowel disease (IBD), especially Crohn's disease (CD). Many CD susceptibility loci have been reported by huge genome-wide linkage-analyses and meta-analyses, i.e., inflammatory bowel disease 5 (IBD5) locus, DLG5, interleukin-23 receptor (IL23R), and autophagy-related 16-like 1 (ATG16L). This study aimed to investigate the possible association of CD with IBD5 gene variants (IGR2198a_1 and IGR2096a_1) and IL23R gene variant (rs1004819) in the Malaysian population. Blood samples from 80 patients diagnosed with CD and 100 controls were collected from the University Malaya Medical Centre (UMMC). DNAs were extracted and analysed by polymerase

Malaysian Symposium

on

Biomedical Science '11

12th-13th of March 2011

Kulliyyah of Science, IIUM Kuantan Campus

"share the knowledge, enlighten the future"

Use Of *stgA* And *spa4289* Genes For Multiplex Polymerase Chain Reaction For Detection Of *Salmonella* Typhi And *Salmonella* Paratyphi A

Mohammad Lukman, Y., Faizul Rahman, S., Nor Amalina, Z., Kenny, D. and Aziah, I.
Institute for Research in Molecular Medicine (INFORMM), Health Campus, Universiti Sains Malaysia

Salmonella Typhi and *Salmonella* Paratyphi A that infect human through fecal-oral route cause typhoid and paratyphoid. Current diagnosis for typhoid and paratyphoid carriers is the identification of the bacteria via culture method and confirmation of the bacteria via biochemical tests and serotyping, which are laborious and time consuming. Therefore the study aims to develop a rapid multiplex PCR test for detection of S. Typhi and S. Paratyphi A. Genomic DNA was isolated form *S. Typhi, S. Paratyphi,* other *Salmonella serovar, Klebsiella pneumonia, Pseudomonas aeruginosa, Acinetobacter baumanni, Escherichia coli* and *Vibrio cholerae*. Mutiplex PCR based on stgA gene encoding for a fimbrial subunit protein and spa4289 gene encoding for DNA methyltransferase of *S.* Paratyphi A was optimized with the annealing temperatures ranging from of 55°C to 75°C. Evaluation of the sensitivity and specificity of the test was performed using *S.* Typhi, *S.* Paratyphi and other organisms. The optimized multiplex PCR for detection of *S.* Typhi and *S.* Paratyphi A was developed with the annealing temperature of 61°C with the amplicon size of 70 bp for *stg*A gene, 93 bp for *spa4289* and 113 bp for IAC. Both sensitivity and specificity of the test were 100%. The analytical sensitivity of the test was 3.13 ng. We conclude that multiplex PCR using stgA and *spa4289* genes has potential to be used for simultaneous detection of *S.* Typhi and *S.* Paratyphi A and further testing will be performed from blood and stool samples of suspected patients.

Keywords: *stgA, spa4289*, multiplex PCR

A Novel Protein for Diagnosis of Amoebic Liver Abscess

Lim Boon Hual, Aziah Ismail, **Tan Chong Leong**
School of Health Sciences, Institute for Molecular medicine, University Sains Malaysia

Amoebic liver abscess (ALA) is an extraintestinal manifestation of *Entamoeba histolytica* infection. Diagnosis of ALA still depends on the commercially available indirect haemagglutination assay (IHA). The kit is expensive and not suitable for use in poor endemic countries. This study aims to explore the efficacy of a previously identified 106 kDa protein of *E. histolytica* HM-1:IMSS for diagnosis of ALA by determining its sensitivity and specificity. The trophozoites cultivated axenically in customized TYI-S-33 medium were used to prepare crude soluble antigen (CSA), which was then used to perform Western blotting. Western blotting was performed using 24 IHA-positive and 30 IHA-negative human serum samples. The sensitivity and

specificity of the protein was shown to be 62.5% and 100% respectively. In conclusion, the 106 kDa protein of *E. histolytica* was shown to be potentially important for diagnosis of ALA.

Keywords: *Entamoeba histolytica*, diagnosis and ALA.

Cloning of Cytochrome P450 2A6 (*CYP2A6*)

Nurul 'Aishah Shaili[1], Nazila Talib[2], W Nur Liana W Mathshor[2], S.M. Mawarni Ramli[2], Hamid Fauzi[2]
[1]*School of Health Sciences and* [2]*Institute for Research in Molecular Medicine (INFORMM), Health Campus, Universiti Sains Malaysia*

Cytochrome P450 2A6 (*CYP2A6*) was first identified as the human coumarin 7-hydroxylase. This enzyme is predominantly expressed in liver and responsible for the clearance of 3% of endogenous and exogenous substances. During the last ten years, *CYP2A6* has received a lot of attention because it has been shown in the principle of human nicotine metabolism which determining an individual smoking behavior and the risk of lung cancer. The objective of this study is to clone *CYP2A6* into expression vector, pEX-C-His. The cDNA of *CYP2A6* was constructed and purchased from ORIGENE USA. The cloning process was performed by using RapidShuttling™ Kit (Origene, USA) and *E. coli* strain BL21 was chosen as the competent cell. Colonies grown were based on ampicillin selection and the insert was screening by Colony PCR. The desired plasmids were extracted by using QIAprep Spin Miniprep Kit from QIAGEN. ABI PRISM 3130xl Genetic Analyzer has been used to analyze the sequence of cDNA. The result showed this *CYP2A6*'s cDNA was successfully cloned into expression vector, pEX-C-His with the sequence of *CYP2A6* was confirmed as in GenBank with accession number of NM_000762.5. In conclusion, the involvement of *CYP2A6* concerning interindividual differences in smoking behavior and the risk of lung cancer is still very unclear, therefore this gene cloning is important for future study in determining an individual susceptibility in lung cancer and also in drug-drug interaction.

Keywords: cloning, *CYP2A6*, drug metabolizing enzymes

Paraoxonase 1 Q192R Polymorphism in Keratoconus and Related Eye Disorders

Yvonne Yong Fong Ling[1], Rozaida Poh Yuen Ying[1], Mary Anne Tan Jin Ai[1], Jenny Parameshvara Deva[2]
[1]*Department of Molecular Medicine, Faculty of Medicine, University of Malaya,* [2]*Tun Hussein Onn National Eye Hospital*

Keratoconus (KC) is an eye disorder due to progressive corneal thinning which leads to corneal coning (Rabinowitz, 1998). Despite intensive studies that have been carried out over the last few decades, the aetiology and pathogenesis of this eye disorder remains unknown. One of the mechanisms postulated was

amdi
Advanced Medical and Dental Institute

INFECTIOUS DISEASE cluster
Advanced Medical & Dental Institute

RECEIVED 15 SEP 2011

USM
UNIVERSITI SAINS MALAYSIA

1st AMDI INTERNATIONAL BIOHEALTH SCIENCE CONFERENCE (IBSC) 2010

Infectious Diseases - Current Challenges
Programme And Abstracts Book

29th November - 1st December 2010
Bayview Beach Resort
Penang, Malaysia.

2010

MB008.O

Cloning, expression and characterization of *Entamoeba histolytica* choline/ethanolamine kinase

Chang Chiat Han[*], *Tan Zi Ning, Few Ling Ling, Lim Boon Huat, See Too Wei Cun*

School of Health Sciences, Universiti Sains Malaysia, Kubang Kerian, Kelantan, Malaysia

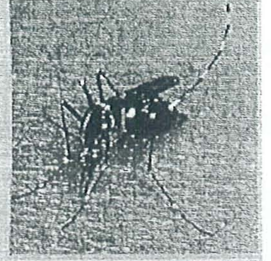
[*]**Corresponding Author**: Chang Chiat Han
E-mail: chiathan@gmail.com

Background/Aim: *Entamoeba histolytica* is one of the most widespread protozoa which causes amoebic liver abscess and intestinal disease. It is the second most death-causing parasite after *Plasmodium falciparum* with 70,000 deaths annually. *E. histolytica* is one of the primitive eukaryote known. Its total lipid is predominated by phospholipid (60-70%) consisting of phosphatidylethanolamine (PtdEtn) and phosphatidylcholine (PtdCho). Phospholipid has been reported to protect *E. histolytica* from self-toxin. Hence, the understanding of PtdCho and PtdEtn biosynthesis is pertinent to control its infection. This study aims to clone, express, purify and characterize *E. histolytica* putative choline/ethanolamine kinase (EhCK/EK), the first enzyme in the main biosynthetic pathway of PtdCho and PtdEtn.

Methodology: Using human choline kinase α1 amino acid sequence as a query, *E. histolytica* putative choline/ethanolamine kinases were identified using NCBI blastp program. Gene candidate was amplified from *E. histolytica* genomic DNA by polymerase chain reaction and inserted into pGEX-RB vector to be expressed as Glutathione S-transferase (GST) fusion EhCK/EK1. The construct was then transformed into *Escherichia coli* BL-21 strain for protein expression. The activity of the purified EhCK/EK1 was shown by spectrophotometric pyruvate kinase-lactate dehydrogenase coupled assay. The mRNA expression of EhCK/EK1 in *E. histolytica* was detected by RT-PCR.

Results: EhCK/EK1 gene was amplified and expressed as a recombinant GST fusion protein. The protein was purified to homogeneity. It was shown to possess activity with choline but not ethanolamine as substrate. RT-PCR result showed that EhCK/EK1 gene was expressed in *E. histolytica*.

Conclusions: We reported the first isolation and characterization of a CK/EK from *E. histolytica*. The enzyme was shown to be a choline-specific kinase and confirmed to be expressed by *E. histolytica*. This enzyme is currently being further studied to characterize its biochemical properties.



PROGRAM AND ABSTRACT BOOK

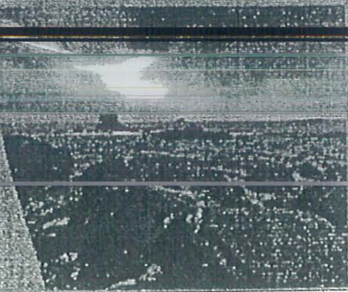
47th Annual Conference of the Malaysian Society of Parasitology & Tropical Medicine

3 & 4 March 2011
IMU Bukit Jalil, Kuala Lumpur Malaysia

Climate Change and its Impact on Public Health

Officiated by
Tan Sri Dato' Dr Abu Bakar Suleiman
President
International Medical University, Kuala Lumpur

Jointly Organised By



PP32

COMPARISON OF *ENTAMOEBA HISTOLYTICA* PROTEIN PROFILES FROM TWO DIFFERENT PREPARATIONS

Lim Boon Huat[1], Tan Zi Ning[1], Wong Weng Kin[2], Foo Phiaw Chong[1] and Rahmah Noordin[2]

[1]*School of Health Sciences, Universiti Sains Malaysia, Malaysia*
[2]*Institute for Research in Molecular Medicine, Universiti Sains Malaysia, Malaysia*

Entamoeba histolytica is an enteric protozoan that causes intestinal amoebiasis, which may manifests into the fatal amoebic liver abscess (ALA) if left untreated. Previous studies reported that membrane-bound proteins of the parasite could be antigenic and play important role in diagnosis. However, some other amoeba species might share similar membrane-bound proteins. Thus, to increase the specificity of diagnosis, membrane-bound proteins can be removed by ether. The aim of this study is to compare the protein profiles of crude soluble antigen (CSA) and ether extract antigen (EEA) of *E. histolytica*. Firstly, 10×10^6 trophozoites were used to prepare either CSA or EEA. CSA was done by sonication technique while EEA was prepared by adding ether to 1X PBS suspended trophozoites and the supernatant was collected after removing the organic phase. Protein concentrations were determined and both CSA and EEA protein profiles were compared by running 12% SDS-PAGE. Several protein bands such as ~40kDa and ~32kDa not found in EEA protein profile were probably located in the plasma membrane. It is interesting to note that both the antigen preparation techniques showed a few prominent protein bands such as ~100kDa, ~45kDa and ~38kDa. Future study will focus on the antigenicities of these proteins.

PP33

PREVALENCE OF TOXOPLASMA GONDII ANTIBODIES IN WOMEN IN TRIPOLI - LIBYA

El-Gomati KM[1], El Naas AS[1], Rashed AM[1] and Elsaid MMA[2]

[1]*Department of Parasitology, Faculty of Veterinary Medicine, Alfateh University, P.O. Box: 13663*
[2]*Department of Parasitolgy, Faculty of Medical technology, Alfateh University, P.O. Box: 13663*

This study carried out to determine the sero-prevalence of *Toxoplasma gondii* antibodies in women. A total of 240 samples of sera form the Obstetric qyna out patient clinic. The sero-positive were 131 out of 240 women (54.58 %). Among the age group 19-28 year the sero-positive 55 (48.25%) out of 114, comparing to the age group 29-38 year and age group 39-48 year were 62 (60.19%) out 103, 14 (60.87%) out of 23 respectively, but statisticaaly no significance according to age. Also found that the women whom are seropositive and had no history of contact with cats were(54.58%), while women whom are seropositive and have history of contact with cats were (57.4%), statistical no significance difference between seropositive, factor contact with cat, and no difference according their residence at different part of Tripoli-Libya.

REC 15 SEP SKB USM

PROGRAM AND ABSTRACT BOOK

47th Annual Conference of the Malaysian Society of Parasitology & Tropical Medicine

3 & 4 March 2011
IMU Bukit Jalil, Kuala Lumpur Malaysia

Climate Change and its Impact on Public Health

Officiated by
Tan Sri Dato' Dr Abu Bakar Suleiman
President
International Medical University, Kuala Lumpur

Jointly Organised By

OP11

RECOGNITION OF POTENTIAL ANTIGENIC PROTEINS FOR DIAGNOSIS OF AMOEBIC LIVER ABSCESS USING TWO DIFFERENT ANTIGEN PREPARATIONS

Oral

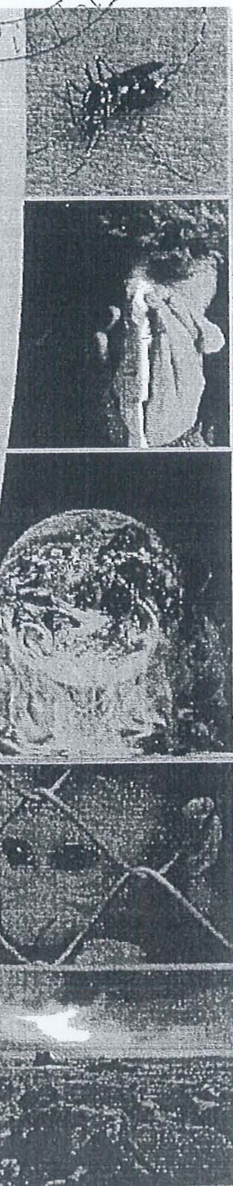
Tan Zi Ning[1], Wong Weng Kin[2], Rahmah Noordin[2] and Lim Boon Huat[1]

[1]School of Health Sciences, Universiti Sains Malaysia, Malaysia
[2]Institute for Research in Molecular Medicine, Universiti Sains Malaysia, Malaysia

Amoebic liver abscess (ALA) is the most common clinical manifestations of amoebiasis in human caused by an enteric protozoan, *Entamoeba histolytica*. This neglected disease has claimed about 100,000 lives and inflicted many more annually. Indirect hemeagglutination assay (IHA), Cellognost ® Amoebiasis (Dade Behring Marburg GmbH, Germany) is frequently used in diagnosis of ALA, but it is costly and not suitable to be used in mass screening of invasive amoebiasis, particularly in endemic developing nations. Thus, the objective of this study is to identify potential antigenic proteins from *E. histolytica* crude soluble antigen (CSA) and ether extract antigens (EEA) for the diagnosis of ALA by using experimentally infected hamster serum samples. Thirty Golden Syrian hamsters were each inoculated with 1 x 10^6 of *E. histolytica* trophozoites to produce ALA. Each animal was sacrificed with 3x overdose of pentobarbital and the cardiac-punctured blood sample was collected to obtain the serum. The CSA was prepared via sonication of trophozoites while the EEA was prepared by solubilizing the trophozoites using ether. Protein concentrations from both antigen preparations were then determined using Bio-Rad Assay. Subsequently, Western blot analysis on both CSA and EEA based on the hamster ALA serum samples revealed that the ~70kDa as a potentially important antigen for diagnosis of hamster ALA. In conclusion, this antigenic protein set the stage for further study in the diagnosis of human ALA.

RECEIVED 15 SEP 2011 SKB USM

Ora



PROGRAM AND ABSTRACT BOOK

47th Annual Conference of the Malaysian Society of Parasitology & Tropical Medicine

3 & 4 March 2011
IMU Bukit Jalil, Kuala Lumpur Malaysia

Climate Change and its Impact on Public Health

Officiated by
Tan Sri Dato' Dr Abu Bakar Suleiman
President
International Medical University, Kuala Lumpur

Jointly Organised By

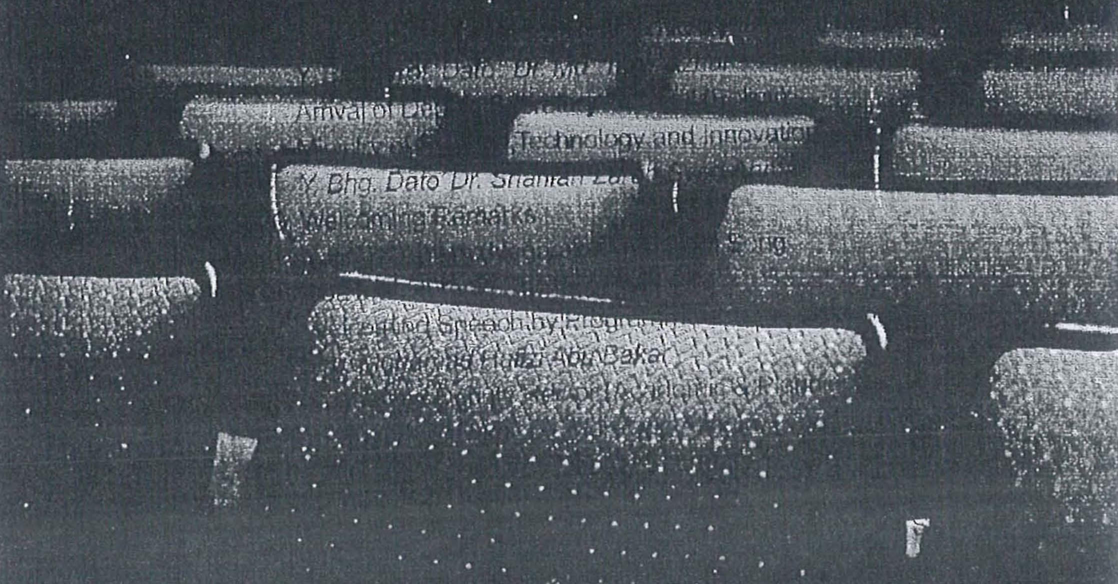
Malaysian Symposium

on

Biomedical Science '11

12th-13th of March 2011

Kulliyyah of Science, IIUM Kuantan Campus



"share the knowledge, enlighten the future"

PROGRAMME SCHEDULE

Preliminary Day (11th March 2011-Friday)

1800 – 2300 Arrival of Participants and Registration
Reception Dinner

Day 1 (12th March 2011-Saturday)

Opening Ceremony, Plenary Talk, Poster Presentation, Career Talk, Career and Research Pathway Exhibition, Bookfair and Forum

0600 – 0700 Subuh Prayer

0730 – 0830 Breakfast
Participant Registration

0830 – 0900 Opening Ceremony
Arrival of participants and students
Arrival of Guests
Arrival of Dean, Kulliyyah of Science, IIUM,
Y. Bhg. Prof. Dr. Kamaruzzaman Yunus
Arrival of Deputy Rector (Academic & Planning) IIUM,
Y. Bhg. Prof. Dato' Dr. Md. Tahir Azhar
Arrival of Deputy Secretary General (Policy),
Ministry of Science,Technology and Innovation,
Y. Bhg. Dato' Dr. Sharifah Zarah Syed Ahmad

0900 – 0920 Welcoming Remarks
National Athem (Negaraku) and IIUM Song
Doa and Quranic Recitation

0920 – 0930 Welcoming Speech by Programme Director, MySympBios 2011,
Bro. Mohamad Hafizi Abu Bakar

0930 – 0945 Speech by Deputy Rector (Academic & Planning), IIUM,
Y. Bhg. Prof. Dato' Dr. Md. Tahir Azhar

0945 – 1005 Officiating Speech by Deputy Secretary General (Policy), MOSTI
Y. Bhg. Dato' Dr. Sharifah Zarah Syed Ahmad

1005 – 1025 Launching on "Malaysian Symposium on Biomedical Science 2011"
Gimmick and Multimedia Presentation

1025 – 1045 Souvenirs Presentation

1045 – 1100 Refreshment for VVIP and VIPs

1100 – 1300 Plenary Talk by Prof. Dr. Syed Mohsin Sahil Jamalullail,
Dean of Research for Biomedical and Health Science Platform, USM
Title: "The Influences of Biomedical Science Area toward Research in Malaysia"

PROGRAMME SCHEDULE

1300 – 1400	Lunch and Zuhr Prayer
1400 – 1630	Final Year Project Competition
	Poster Presentation
	Career and Research Pathway Exhibition
	Bookfair
	Career Talk 1 "Bioethics in Biomedical Research"
	Career Talk 2 "Career in Biomedical Research: The Challenge & Reward"
	Career Talk 3 "Career in NIOSH: The Challenge & Reality"
1630 – 1700	Tea-break
1700 – 1900	Forum on "Biomedical Science: Past, Present and Future, Finding a Bridge"
1900	Disperse
2100 – 2300	BBQ Dinner
2300	Disperse

Day 2 (13th March 2011-Sunday)

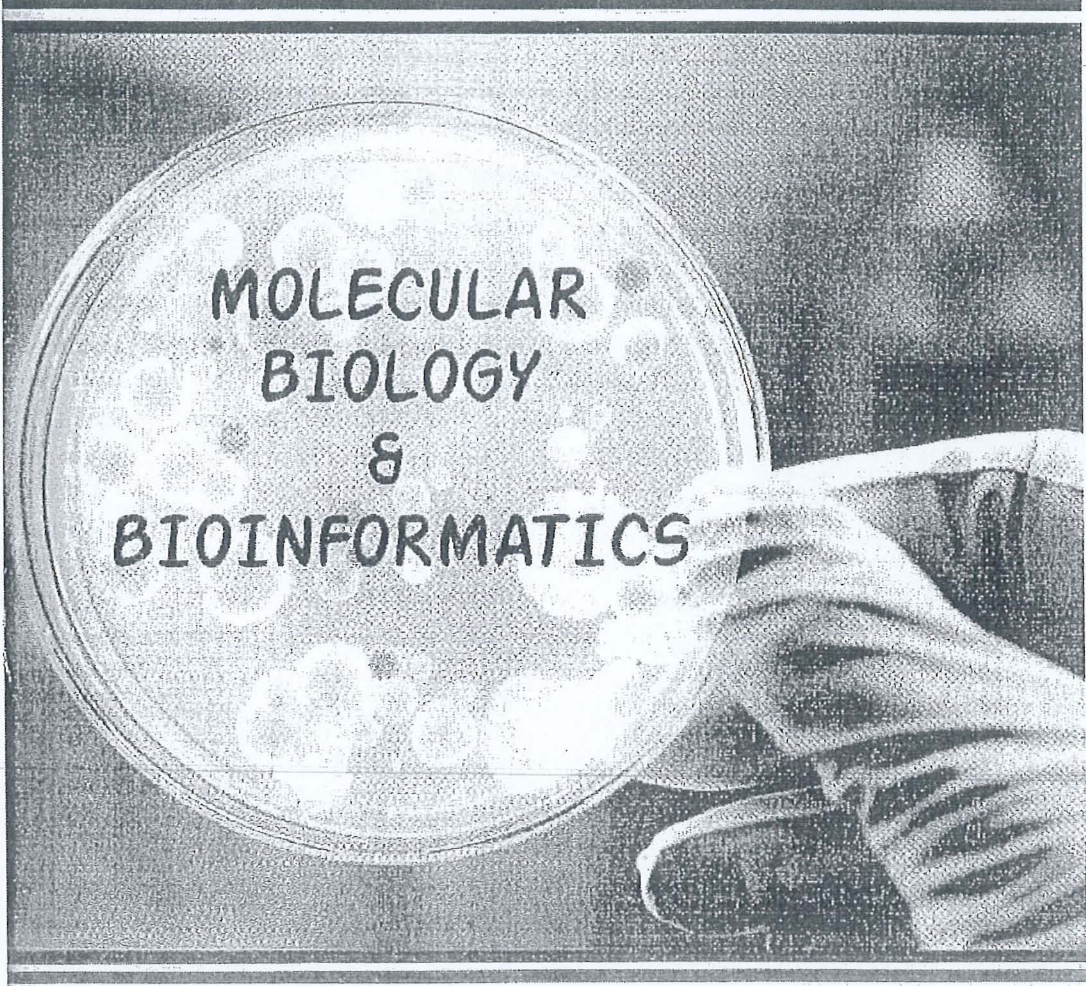
Oral and Poster Presentation, Career and Research Pathway Exhibition, Bookfair, Keynote Sessions and Closing Ceremony

0600 – 0700	Subuh Prayer
0700 – 0830	Arrival of Participants
	Briefing
	Breakfast
0900 – 1230	Talk by Keynote Speakers
	Microbiology, Immunology and Cell Biology:
	Assoc. Prof. Dr. Mohammad Tariqur Rahman
	Pharmacology, Toxicology and Biochemistry:
	Prof. Dr. Mohammad b.Osman
	Career and Research Pathway Exhibition
	Bookfair
	Final Year Project Competition
	Oral Presentation Session 1
	Poster Presentation
1230 – 1400	Lunch
	Zuhr Prayer

PROGRAMME SCHEDULE

1400 – 1630	Talk by Keynote Speakers Anatomy and Physiology: *Asst. Prof. Dr. Munirah Sha'ban* Molecular Biology and Bioinformatics: *Assoc. Prof. Dr. Shaharum Shamsuddin* Career and Research Pathway Exhibition Bookfair Final Year Project Competition Oral Presentation Session 2
1630 – 1700	Refreshment 'Asr Prayer
1700 – 1800	Closing Ceremony Arrival of Participants Arrival of Guests Arrival of Chairman, Malaysian Symposium on Biomedical Science 2011, *Madam Norazsida Ramli* Arrival of Dean, Kulliyyah of Science, International Islamic University Malaysia *Prof. Dr. Kamaruzzaman Yunus*
1800 – 1820	Closing Remarks National Anthem (Negaraku) & IIUM Song
1820 – 1825	Speech by Chairman, MySympBios 2011, *Madam Norazsida Ramli*
1825 – 1835	Closing Speech by Dean, Kulliyyah of Science, IIUM, *Prof. Dr. Kamaruzzaman Yunus*
1835 – 1900	Presentation of MySympBios 2011 Awards (Oral and Poster) Presentation of Participants Certificates Announcing the next host for Malaysian Symposium on Biomedical Science 2012
1900 – 1920	Recitation of Du'a Closing Remarks Group Photography Session
1920 – 2000	Disperse Maghrib Prayer
2000 – 2030	Dinner
2030 – 2200	Participants check out

ORAL SESSION


MOLECULAR
BIOLOGY
&
BIOINFORMATICS

Potentially important antigen of *Entamoeba histolytica* for diagnosis of amoebic liver abscess: An animal model

Lim Boon Huat[1], Aziah Ismail[2], **Siti Shafiqah Anaqi Azham**[1]
School of Health Sciences[1], Institute for Molecular medicine[2], University Sains Malaysia

Entamoeba histolytica is a causative agent of amoebiasis. Most of the infections are asymptomatic while others range from dysentery to amoebic liver abscess (ALA), which is potentially fatal. The commercially available indirect haemagglutination assay (IHA) is the common detection method for ALA. The assay is expensive, thus is not suitable for use in poor endemic countries. This study aimed to identify potentially important antigen(s) of *E. histolytica* based on the ALA-positive serum samples of Golden Syrian hamsters. The crude soluble antigen (CSA) of *E. histolytica* was prepared from axenic culture of the amoeba strain. After that, SDS-PAGE protein profiling was performed, followed by Western blot analysis. Thirty-two infected hamster serum samples and 7 healthy hamster serum samples were used as the primary antibody, and Tris-buffered saline (TBS) was used as the negative control. Anti-hamster antibody was used as the secondary antibody and tetramethylbenzene (TMB) was used as the detection reagent. The molecular weight of antigenic protein bands were analyzed by using gel pro analyzer. The results showed several antigenic bands with different molecular weights ranging from 70 kDa to 130 kDa. The 97 kDa protein band showed a sensitivity and specificity of 91% and 100% respectively. In conclusion, the protein band was found to be potentially important for detection of ALA in human.

Keywords: Entamoeba histolytica, Diagnosis and Amoebic liver abscess.

Genetic Polymorphisms Of The Inflammatory Bowel Disease 5 (*IBD5*) And Interleukin-23 Receptor (*IL23R*) Genes In Malaysian Crohn's Disease Patients

Chua Kek Heng, Lian Lay Hoong, **Sathya Narayanan A/L Patmanathan**
Department of Molecular Medicine, University of Malaya

There are strong evidences that support the role of genetic factors in the susceptibility of individuals to inflammatory bowel disease (IBD), especially Crohn's disease (CD). Many CD susceptibility loci have been reported by huge genome-wide linkage-analyses and meta-analyses, i.e., inflammatory bowel disease 5 (IBD5) locus, DLG5, interleukin-23 receptor (IL23R), and autophagy-related 16-like 1 (ATG16L). This study aimed to investigate the possible association of CD with IBD5 gene variants (IGR2198a_1 and IGR2096a_1) and IL23R gene variant (rs1004819) in the Malaysian population. Blood samples from 80 patients diagnosed with CD and 100 controls were collected from the University Malaya Medical Centre (UMMC). DNAs were extracted and analysed by polymerase

Malaysian Symposium

on

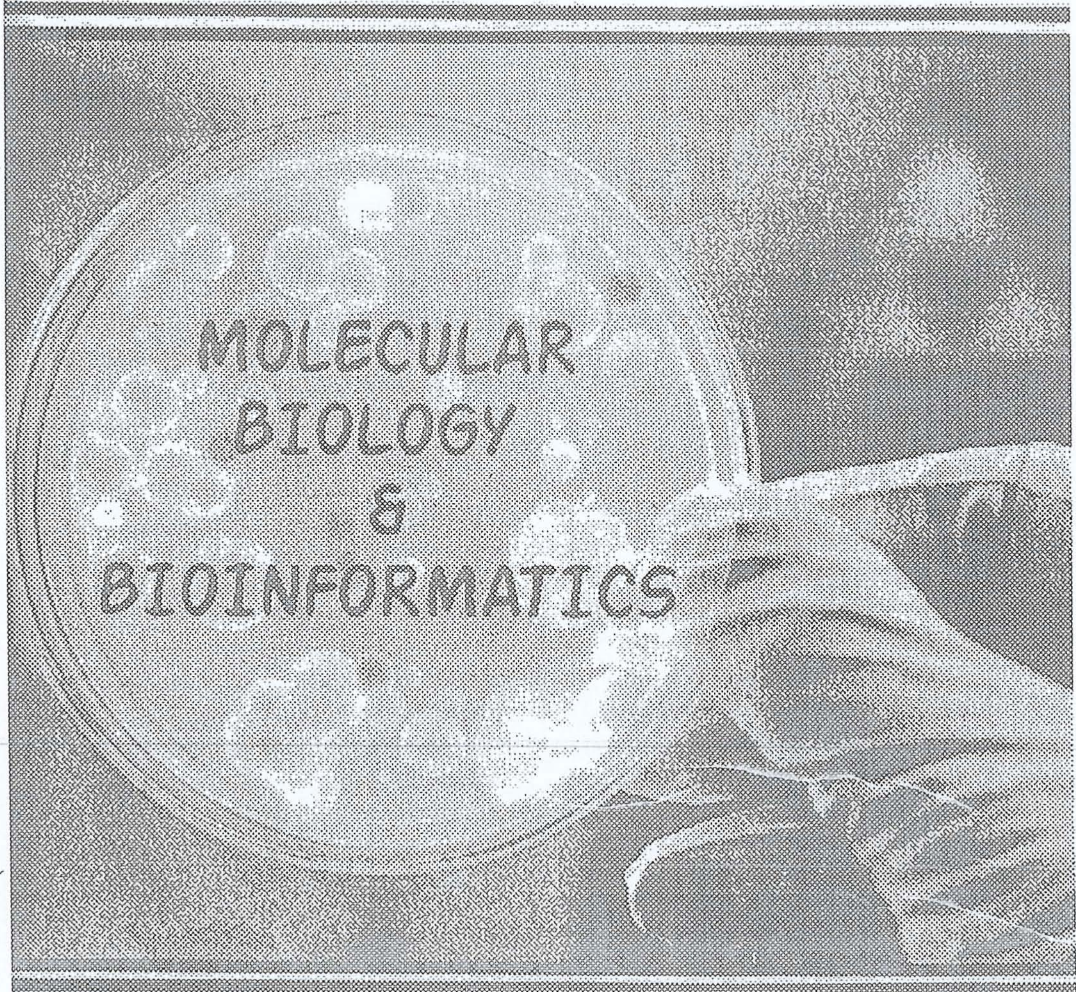
Biomedical Science '11

12th-13th of March 2011

Kulliyyah of Science, IIUM Kuantan Campus

"share the knowledge, enlighten the future"

POSTER
SESSION


MOLECULAR
BIOLOGY
&
BIOINFORMATICS

Use Of *stgA* And *spa4289* Genes For Multiplex Polymerase Chain Reaction For Detection Of *Salmonella* Typhi And *Salmonella* Paratyphi A

Mohammad Lukman, Y., Faizul Rahman, S., Nor Amalina, Z., Kenny, D. and Aziah, I.
Institute for Research in Molecular Medicine (INFORMM), Health Campus, Universiti Sains Malaysia

Salmonella Typhi and *Salmonella* Paratyphi A that infect human through fecal-oral route cause typhoid and paratyphoid. Current diagnosis for typhoid and paratyphoid carriers is the identification of the bacteria via culture method and confirmation of the bacteria via biochemical tests and serotyping, which are laborious and time consuming. Therefore the study aims to develop a rapid multiplex PCR test for detection of S. Typhi and S. Paratyphi A. Genomic DNA was isolated form *S. Typhi*, *S. Paratyphi*, other *Salmonella serovar*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Acinetobacter baumanni*, *Escherichia coli* and *Vibrio cholerae*. Multiplex PCR based on stgA gene encoding for a fimbrial subunit protein and spa4289 gene encoding for DNA methyltransferase of *S.* Paratyphi A was optimized with the annealing temperatures ranging from of 55°C to 75°C. Evaluation of the sensitivity and specficity of the test was performed using *S.* Typhi, *S.* Paratyphi and other organisms. The optimized multiplex PCR for detection of *S.* Typhi and *S.* Paratyphi A was developed with the annealing temperature of 61°C with the amplicon size of 70 bp for stgA gene, 93 bp for *spa4289* and 113 bp for IAC. Both sensitivity and specificity of the test were 100%. The analytical sensitivity of the test was 3.13 ng. We conclude that multiplex PCR using stgA and *spa4289* genes has potential to be used for simultaneous detection of S. Typhi and S. Paratyphi A and further testing will be performed from blood and stool samples of suspected patients.

Keywords: *stgA*, *spa4289*, multiplex PCR

A Novel Protein for Diagnosis of Amoebic Liver Abscess

poster

Lim Boon Huat, Aziah Ismail, **Tan Chong Leong**
School of Health Sciences, Institute for Molecular medicine, University Sains Malaysia

Amoebic liver abscess (ALA) is an extraintestinal manifestation of *Entamoeba histolytica* infection. Diagnosis of ALA still depends on the commercially available indirect haemagglutination assay (IHA). The kit is expensive and not suitable for use in poor endemic countries. This study aims to explore the efficacy of a previously identified 106 kDa protein of *E. histolytica* HM-1:IMSS for diagnosis of ALA by determining its sensitivity and specificity. The trophozoites cultivated axenically in customized TYI-S-33 medium were used to prepare crude soluble antigen (CSA), which was then used to perform Western blotting. Western blotting was performed using 24 IHA-positive and 30 IHA-negative human serum samples. The sensitivity and

specificity of the protein was shown to be 62.5% and 100% respectively. In conclusion, the 106 kDa protein of *E. histolytica* was shown to be potentially important for diagnosis of ALA.

Keywords: *Entamoeba histolytica*, diagnosis and ALA.

Cloning of Cytochrome P450 2A6 (*CYP2A6*)

Nurul 'Aishah Shafii[1], Nazila Talib[2], W Nur Liana W Mathshor[2], S.M. Mawarni Ramli[2], Hamid Fauzi[2]
[1]*School of Health Sciences and* [2]*Institute for Research in Molecular Medicine (INFORMM), Health Campus, Universiti Sains Malaysia*

Cytochrome P450 2A6 (*CYP2A6*) was first identified as the human coumarin 7-hydroxylase. This enzyme is predominantly expressed in liver and responsible for the clearance of 3% of endogenous and exogenous substances. During the last ten years, *CYP2A6* has received a lot of attention because it has been shown in the principle of human nicotine metabolism which determining an individual smoking behavior and the risk of lung cancer. The objective of this study is to clone *CYP2A6* into expression vector, pEX-C-His. The cDNA of *CYP2A6* was constructed and purchased from ORIGENE USA. The cloning process was performed by using RapidShuttling™ Kit (Origene, USA) and *E. coli* strain BL21 was chosen as the competent cell. Colonies grown were based on ampicillin selection and the insert was screening by Colony PCR. The desired plasmids were extracted by using QIAprep Spin Miniprep Kit from QIAGEN. ABI PRISM 3130xl Genetic Analyzer has been used to analyze the sequence of cDNA. The result showed this *CYP2A6*'s cDNA was successfully cloned into expression vector, pEX-C-His with the sequence of *CYP2A6* was confirmed as in GenBank with accession number of NM_000762.5. In conclusion, the involvement of *CYP2A6* concerning interindividual differences in smoking behavior and the risk of lung cancer is still very unclear, therefore this gene cloning is important for future study in determining an individual susceptibility in lung cancer and also in drug-drug interaction.

Keywords: cloning, *CYP2A6*, drug metabolizing enzymes

Paraoxonase 1 Q192R Polymorphism in Keratoconus and Related Eye Disorders

Yvonne Yong Fong Ling[1], Rozaida Poh Yuen Ying[1], Mary Anne Tan Jin Ai[1], Jenny Parameshvara Deva[2]
[1]*Department of Molecular Medicine, Faculty of Medicine, University of Malaya,* [2]*Tun Hussein Onn National Eye Hospital*

Keratoconus (KC) is an eye disorder due to progressive corneal thinning which leads to corneal coning (Rabinowitz, 1998). Despite intensive studies that have been carried out over the last few decades, the etiology and pathogenesis of this eye disorder remains unknown. One of the mechanisms postulated was

1 **Analysis of *Entamoeba histolytica* excretory-secretory antigen (ESA) and**

2 **identification of a new potential diagnostic marker**

3 **Running title: *Entamoeba histolytica* ESA of diagnostic potential**

4

5 Wong Weng Kin,[1] Tan Zi Ning,[2] Nurulhasanah Othman,[1] Lim Boon Huat,[2] Zeehaida

6 Mohamed,[3] Alfonso Olivos Garcia,[4] and Rahmah Noordin[1*]

7

8 Institute for Research in Molecular Medicine, Universiti Sains Malaysia, 11800, Penang

9 Malaysia[1],

10 School of Health Sciences, Universiti Sains Malaysia, 16150, Kelantan, Malaysia[2],

11 Department of Medical Microbiology and Parasitology, School of Medical Sciences,

12 Universiti Sains Malaysia, 16150, Kelantan, Malaysia[3],

13 Departmento de Medicina Experimental, Facultad de Medicina, Universidad Nacional

14 Autónoma de México 04510 Mexico D.F., Mexico[4]

15

16

17

18

19

*Corresponding author. Mailing address: Institute for Research in Molecular Medicine, Universiti Sains Malaysia, 11800, Penang, Malaysia. Phone: 604-6534802. Fax: 604-6534813. E-mail: rahmah8485@gmail.com.

20 **ABSTRACT**

21 Serodiagnosis of amoebiasis remains the preferred method for diagnosis of ALA.
22 However, the commercially available kits are problematic in endemic areas due to the
23 persistent high background antibody titers. Human serum samples (n=38) from patients
24 with amoebic liver abscess (ALA) who live in endemic areas were collected from
25 Hospital Universiti Sains Malaysia during the period of 2008-2010. Western blot analysis
26 using excretory-secretory antigen (ESA) collected from axenically grown *E. histolytica*
27 were probed with the above serum samples. Seven antigenic proteins of ESA with
28 varying reactivities were identified *i.e.* 152 kDa, 131 kDa, 123 kDa, 110 kDa, 100 kDa,
29 82 kDa and 76 kDa. However, only 152 kDa and 110 kDa proteins showed sensitivities
30 above 80 % in the Western blot analysis. All the antigenic proteins showed undetectable
31 cross-reactivity when probed with healthy human serum samples (n=30) and serum
32 samples from other infections (n=33). From the MALDI-TOF-TOF analysis, the proteins
33 were identified as heavy subunit of *E. histolytica* lectin and *E. histolytica* pyruvate
34 phosphate dikinase, respectively. Use of the *E. histolytica* lectin for diagnosis of ALA has
35 been well reported by researchers and is being used in commercialized kits. However,
36 this is the first report on the potential use of pyruvate phosphate dikinase for diagnosis of
37 ALA, thus this molecule merits further evaluation on its diagnostic value using a larger
38 panel of serum samples.

39

40 # INTRODUCTION

41

42 Amoebiasis is caused by the enteric protozoan *Entamoeba histolytica*, which
43 affects 50 million of the world population and leads to 100,000 fatal cases annually (17,
44 18). Amoebic liver abscess (ALA) is the most common clinical manifestation of
45 extraintestinal amoebiasis. It is due to the haematogenous spread of the *E. histolytica*
46 trophozoites from intestine to liver through the portal vein. Patients with ALA present
47 with hepatomegaly, right upper quadrant pain, tenderness of the liver, fever, jaundice and
48 nausea. It may lead to fatal outcome if early diagnosis and treatment are not sought (1,
49 10).

50

51 Diagnosis of ALA is often initiated with radiology imaging to examine the
52 presence of abscess in the liver. If indicated, aspiration of the sample is performed for
53 culture, DNA detection and/or antigen detection. Absence of bacteria growth in the
54 abscess culture could rule out the possibility of pyogenic liver abscess cases. The
55 definitive diagnosis of ALA is by microscopic observation of trophozoites in the abscess
56 fluid, but the sensitivity of microscopic examination is low as the trophozoites are easily
57 disintegrated and most of them reside at the peripheral margin of the abscess.
58 Nevertheless, it is unethical to perform abscess aspirate close to the peripheral margin of
59 abscess, as this may damage the healthy liver tissues. Many reports showed that DNA
60 and antigen detection-based methods performed on the abscess sample *e.g.* PCR, real-
61 time PCR, Techlab *E. histolytica* II antigen detection ELISA, gave high sensitivity (4, 9,
62 16).

63

64

65 Whenever abscess aspiration cannot be performed due to small abscess size or
66 bad condition of the patient, serological test becomes the preferred choice for diagnosis.
67 The available antigen detection tests such as Techlab *E. histolytica* II ELISA, which
68 detects *E. histolytica* lectin antigen, can be used for diagnosis of acute ALA patient who
69 have not received treatment (21). However often patients who are admitted to the hospital
70 with liver abscess have received treatment prior to investigation for ALA, which
71 significantly reduces the sensitivity of the antigen detection test. Thus antibody detection
72 is currently the most common serological test used to detect ALA, either by indirect
73 haemagglutination assay (IHA) or ELISA. However, these tests mostly use amoebic
74 lysate antigen, and are problematic for diagnosis in endemic area where the background
75 anti-amoebic antibody titer is high. Thus in endemic areas, low specificities of these tests
76 were reported with the low cut-off values as suggested by the manufacturer (20, 22).

77

78 Comparison of crude soluble antigen (CSA) with excretory secretory antigen
79 (ESA) of *E. histolytica* have been shown to demonstrate higher positive detection rate
80 when tested with sera of patients with acute amoebic dysentery and asymptomatic cysts
81 passer, and equal sensitivity for diagnosis of ALA (8, 13). Therefore, in our quest to
82 identify new markers to improve the serodiagnosis of ALA, ESA of *E. histolytica* was
83 produced and analysed by SDS-PAGE, two-dimensional electrophoresis (2-DE) and
84 Western blot. The identities of the potential candidates were then identified by mass-
85 spectrometry.

86

87 ## MATERIALS AND METHODS

88

89 **Maintenance of *E. histolytica* trophozoites.** Axenic strain *E. histolytica*
90 trophozoites HM1:IMSS was hermetically cultured in TYI-S-33 medium supplemented
91 with 12.5 % bovine serum (Invitrogen, New Zealand) and 1X Diamond vitamin Tween-
92 80 (Sigma, USA) at 36 °C. The medium was changed every 48-72 hours (2).

93

94 **Collection and preparation of ESA.** Mass culture of *E. histolytica* trophozoites
95 were collected at log phase and washed 3X with RMPI supplemented with 0.1 % L-
96 cysteine and 0.02 % ascorbic acid (RPMI-C&A) with centrifugation at 22 x g for 2 min,
97 RT. Subsequently, the cell density was determined via trypan blue exclusion method.
98 Trophozoites were seeded into culture tube containing 80 % filled RMPI-C&A medium
99 with cell density of 0.8 x 10^6 cells per mL and incubated at 36 °C for 6 hours. Upon
100 completion, culture tubes were subjected to centrifugation at 22 x g for 2 min at 4 °C.
101 The supernatant in the culture tubes were collected and mixed with 0.5 M iodoacetamide
102 to a final concentration of 1 mM. Next, the supernatant was again subjected to
103 centrifugation at 10, 000 x g for 5 min at 4 °C and filtered through 0.2 μm membrane.
104 Subsequently, ESA in the supernatant was concentrated 1000X using U-Tube
105 concentrator with MWCO of 10 kDa. Cocktail protease inhibitor (Roche Diagnostic,
106 Germany) was then added to the concentrated ESA. The protein concentration of the ESA
107 was estimated using Bradford protein assay (8, 13, 19).

108

109 **Serum samples and ethical approval.** Serum samples in the current study were
110 collected from Hospital Universiti Sains Malaysia during the period of 2008-2010. The
111 procedures of collecting and handling the serum samples were approved by USM Human
112 Ethical Committee (Ref. No.: USMKK/PPP/JEPem[213.3(10)]). Human serum samples
113 included in this study were divided into four groups: (i) Group A: human ALA serum
114 samples (n=24) with consistent clinical symptoms (*i.e.* fever, abdominal/right hepatic
115 chest pain, hepatomegaly, and jaundice) and radiological image, and IHA positive results;
116 (ii) Group B: human ALA serum samples (n=14) from patients whose abscess were
117 positive by real-time PCR for *E. histolytica* DNA and negative by bacterial culture; (iii)
118 Group C: healthy blood donor serum samples which were negative by IHA (n=30); (iv)
119 Group D: serum samples from patients with other infections (n=33) *i.e.* salmonellosis
120 (n=5), shigellosis (n=1), *Escherichia coli* septicaemia (n=2), *Staphylococcus* spp.
121 septicaemia (n=2), *H. pylori* (n=6), pyogenic liver abscess (n=4), *Stenotrophomonas*
122 *maltophilia* septicaemia (n=1), Enteropathogenic *Escherichia coli* (n=1), *Ascaris*
123 *lumbricoides* (n=1), *Klebsiella pneumoniae* (n=1) and toxoplasmosis (n=9). These sera
124 were negative by IHA for amoebiasis.
125
126 **SDS-PAGE.** Protein samples were electrophoretically separated via SDS-PAGE
127 using Bio-Rad Mini Protean III Electrophoresis Cell and Protean® II xi Cell according to
128 Laemmli (7) protocol with modifications. Prior to SDS-PAGE, ESA was mixed with 2X
129 Laemmli sample buffer and boiled for 5 min. Subsequently, it was separated using 6%
130 SDS-PAGE gel, at constant current of 25 mA per gel for about 1 h.
131

132 **Western blotting.** Upon completion of SDS-PAGE, proteins in the gel was
133 electrophoretically transferred onto a 0.45 μm nitrocellulose membrane (NCP) using
134 semi-dry transblot (Bio Rad, USA) at a constant voltage of 15 V for 30 min. The NCP
135 was blocked for 1hr at RT with 5 % skim milk prepared in 10 mM Tris buffered saline,
136 pH 7.2 (TBS). Subsequently, the NCP was washed (3 x 5 min) with TBS containing 0.1
137 % Tween-20 (TBS-T). Then, the NCP was cut into multiple strips and incubated with
138 human sera at dilution of 1:200 (in TBS-T) for 2 hours at RT. The NCP strips were then
139 washed three times with TBS-T, and then incubated with monoclonal mouse anti–human
140 IgG conjugated with horseradish peroxides (HRP) at dilution of 1:6000 for 1hr.
141 Subsequently, the NCP strips were again washed (3 x 5 min) with TBS-T. Western blot
142 substrates *i.e.* enhanced chemiluminescence (ECL) blotting reagent (Roche diagnostics,
143 Germany) or tetramethylbenzidine (TMB) substrate for membrane (Sigma, USA) were
144 usedas substrates. The Western blot signal was captured using camera (Lumix,
145 Germany).
146

147 **2-DE and Western blot.** Selected protein bands which showed potential
148 diagnostic value were further analysed using 2-DE to ensure that the bands were well-
149 separated. OFFGEL fractionator 3100 (Agilent Technologies, Germany) was used to
150 separate the proteins by isoelectric points (pI) followed by SDS-PAGE and Western blot.
151 Agilent OFFGEL Kit pH 3–10 with a 12-well setup frame was used. Sample was
152 prepared by mixing 1600 μl of the ready stock solution (1.25X) with 400 μl of the sample
153 with total protein amount of 2 mg and then gently vortexed. Forty microliter of IPG strip
154 rehydration buffer was added into each well to swell the gel for 15 minutes. Wetted
155 electrode pads were placed at the cathode- and anode-ends of the IPG strip gel surface.
156 After re-swelling of the gel, 150 μL of protein sample was loaded into each well. Ten
157 microliter of rehydration buffer was reapplied onto the electrode pads at each of the IPG
158 gel ends. Cover fluid (mineral oil) was pipetted onto the gel strip ends. Subsequently, the
159 sample was focused with a maximum power of 200 mW, maximum current of 50 mA and
160 typical voltages ranging from 500 to 4500 V until 50 kVh was reached after 24 h. Upon
161 completion, each of the twelve fractionated ESA samples were separately mixed with 5X
162 Laemmli sample buffer without boiling and electrophoretically separated via SDS-
163 PAGE.Western blot was performed using pooled and individual human serum samples to
164 identify the selected antigenic proteins.
165
166 **Mass spectrometry analysis & protein Identification.** The selected proteins
167 were excised from 2D-SDS-PAGE gel and sent for MALDI-TOF-TOF (4800) analysis at
168 Proteomic Laboratory Service Center, Australia, and searched with Swiss-Prot protein
169 database.

170 ## RESULTS

171

172 **IgG blots of ESA.** IgG blots of ESA probed with human ALA serum samples
173 from Group A showed seven antigenic bands with consistent reactivities (Fig.1). Besides,
174 these antigenic proteins were also similar with the bands present in the IgG blots probed
175 with human ALA serum samples from Group B (Fig. 2). However, mean sensitivities of
176 the bands to detect ALA vary from 16 % to 84 %. Only two of the antigenic bands *i.e.*
177 152 kDa and 110 kDa showed high sensitivities of about 80 % in both Groups A and B
178 sera (Table 1). Neither of these two antigenic bands showed reactivity in IgG blots
179 probed with serum samples from Groups C and D, thus showing 100 % specificity.

180

181 **2-DE Western blot and protein identification.** IgG blot of 12 ESA fractions
182 with pooled serum sample revealed that the 152 kDa and 110 kDa proteins were located
183 in Fraction 5 (pI: 5.33-5.91) and Fraction 6 (pI: 5.91-6.5), respectively (Fig. 3). Further
184 IgG blot analysis of these ESA fractions with individual serum samples (n=5) confirmed
185 the location of these antigenic proteins (Fig. 4). These protein bands were excised and
186 sent for MALDI-TOF-TOF analysis. According to Mascot search result from MSDB
187 search engine, the 152 kDa protein matched with *E. histolytica* lectin protein (C4LTMO)
188 with the protein score of 273. A score >55 indicates identity or extensive homology at a
189 significant level ($p < 0.05$). Seven peptides matched to the Gal/GalNAc lectin heavy
190 subunit. The 110 kDa protein matched with pyruvate phosphate dikinase (EHI_009530)
191 with the protein score of 544, with nine matched peptides.

192

193 ## DISCUSSION

194 Current diagnosis of ALA still depends on the results of clinical manifestations,
195 radiology imaging and laboratory tests, since stool examination is inapplicable for
196 diagnosis of extraintestinal amoebiasis. Detection of *E. histolytica*-specific DNA and
197 antigen in the aspirated abscess are more sensitive and specific, as compared to
198 microscopic examination of live trophozoites. However, in most settings when abscess
199 aspiration cannot be performed, serological test is the alternative laboratory method for
200 diagnosis of ALA. Besides being relatively less invasive, it is also less technical
201 demanding and thus is routinely used in most diagnostic laboratories.

202

203 *E. histolytica* ESA contains proteins shed from trophozoites during active
204 multiplication and metabolites released by trophozoites during incubation in RPMI-C&A.
205 Although great care was taken to produce good quality ESA, there were probably still
206 some partial proteins released from lysed trophozoites.In this study, the ESA antigenic
207 bands that ranged from 97.2-158 kDa consistently showed reactivities when incubated
208 with human ALA serum samples (n=7). Analysis of the IgG blots showed that 152 kDa
209 and 110 kDa proteins had higher association with serum samples from patients with
210 PCR-positive abscess (93 % and 86 %) as compared to serum samples from patients with
211 unknown PCR results (both 79 %). Besides, the sensitivity of both 152 kDa and 110 kDa
212 were similar, *i.e.* 84 % and 82 %, respectively. Both antigens showed high specificity as
213 there were undetectable reactivities in the IgG blot of ESA probed with serum samples
214 from normal individuals and those with other infections.

215

216 The protein components of ESA in the current study were different from those
217 reported by Sengupta *et al.*(13). In the latter study, the ESA proteins ranged between 200-
218 20 kDa, with predominant protein bands below 66 kDa (*e.g.* 45 kDa and 29 kDa), while
219 the high molecular weight proteins (> 100 kDa) were faint. This may be due to the
220 differences in antigen preparations. The ESA in this study was concentrated 1000X
221 instead of 10X to enrich the low abundant proteins. In addition, to enhance the
222 reproducibility of ESA, protein-free defined RPMI-C&A was used in this study, instead
223 of serum- and vitamin-free TYI-S-33. Besides, iodoacetamide was added to the RPMI-
224 C&A containing the ESA upon its collection, in order to protect the protein from
225 degradation by proteases (3, 11, 13).
226
227 2-DE protein separation *via* Agilent 3100 OFFGEL Fractionator followed by
228 SDS-PAGE allowed only the selected ESA fractions to be tested with serum samples.
229 The protein bands excised from the 2-DE gels were well-separated, thus avoiding the
230 presence of multiple proteins in each band. In this study, the 152 kDa protein was
231 identified as *E. histolytica* lectin protein, which has been reported to be sensitive for
232 diagnosis of invasive amoebiasis (5, 6). Specific monoclonal antibody against this protein
233 has been used for antigen detection test in TechLab Entamoeba histolytica II kit
234 (TechLab Inc, USA). The 110 kDa protein, identified as the *E. histolytica* pyruvate
235 phosphate dikinase, was also found to show similar high sensitivity for diagnosis of ALA.
236 This protein was reported to be a key enzyme in the anaerobic metabolism *via*
237 pyrophosphate dependent glycolysis, and has no counterpart with proteins in human
238 metabolism(12). Molecular modeling of this enzyme had been reported, and specific

239 inhibitors to it for therapeutic purpose have been studied (15). The protein sequence of
240 pyruvate phosphate dikinase showed high similarity with a closely related pathogenic
241 intestinal anaerobic protozoa *i.e. Giardia lamblia*; this suggests the possibility of
242 producing specific antibody for simultaneous detection of both species, as well as for
243 differential detection (14). To date, there is no report on the application of pyruvate
244 phosphate kinase for diagnosis of amoebiasis. Further studies on this protein will be
245 performed, which include production of the recombinant form of the protein and testing
246 with a larger panel of serum samples.

247

248 **ACKNOWLEDGEMENTS**

249 This study was funded by FRGS grant No. 203/CIPPM/6711122, USM-RU Grant
250 No. 1001/PPSK/813009, and USM-RU-PGRS Grant No. 1001/INFORMM/8032030. The
251 first author is a recipient of USM Fellowship, and the third author received financial
252 support from the Vice Chancellor's Award.

253

254 **REFERENCES**

255 1. **Akgun, Y., I.H. Tacyildiz, and Y. Celik.** 1999. Amebic liver abscess: changing
256 trends over 20 years. World J. Surg. **23:**102-106.
257 2. **Diamond, L.S., D.R. Harlow, and C.C. Cunnick.** 1978. A new medium for the
258 axenic cultivation of *Entamoeba histolytica* and other *Entamoeba.* Trans R. Soc.
259 Trop. Med. Hyg. **72:**431-432.
260 3. **Flores, M.S., et al.** 2005. Preparation of *Entamoeba histolytica* antigens without
261 enzymatic inhibitors. Parasitology. **131:**231-236.

262 4. **Fotedar, R., et al.** 2007. Laboratory diagnostic techniques for *Entamoeba* species.
263 Clin. Microbiol. Rev. **20:**511-532.
264 5. **Haque, R., et al.** 1997. *Entamoeba histolytica* and *Entamoeba dispar* infection in
265 children in Bangladesh. The Journal of infectious diseases. **175:**734-736.
266 6. **Haque, R. and W.A. Petri, Jr.** 2006. Diagnosis of amebiasis in Bangladesh.
267 Arch. Med. Res. **37:**273-276.
268 7. **Laemmli, U.K.** 1970. Cleavage of structural proteins during the assembly of the
269 head of bacteriophage T4. Nature **227:**680-685.
270 8. **Pal, S., et al.** 1996. Comparative evaluation of somatic & excretory-secretory
271 antigens of *Entamoeba histolytica* in serodiagnosis of human amoebiasis by
272 ELISA. Indian J. Med. Res. **104:**152-156.
273 9. **Paul, J., S. Srivastava, and S. Bhattacharya.** 2007.Molecular methods for
274 diagnosis of *Entamoeba histolytica* in a clinical setting: an overview. Exp.
275 Parasitol. **116:**35-43.
276 10. **Petri, W.A., Jr. and U. Singh.** 1999.Diagnosis and management of amebiasis.
277 Clin. Infect. Dis. **29:**1117-1125.
278 11. **Que, X. and S.L. Reed.** 2000. Cysteine proteinases and the pathogenesis of
279 amebiasis. Clin. Microbiol. Rev. **13:**196-206.
280 12. **Saavedra-Lira, E., L. Ramirez-Silva, and R. Perez-Montfort.** 1998.
281 Expression and characterization of recombinant pyruvate phosphate dikinase from
282 *Entamoeba histolytica.* Biochim. Biophys. Acta. **1382:**47-54.
283 13. **Sengupta, S., et al.** 2000. Role of excretory-secretory products of *Entamoeba*
284 *histolytica* in human amebiasis. Arch. Med. Res. **31:**226-228.

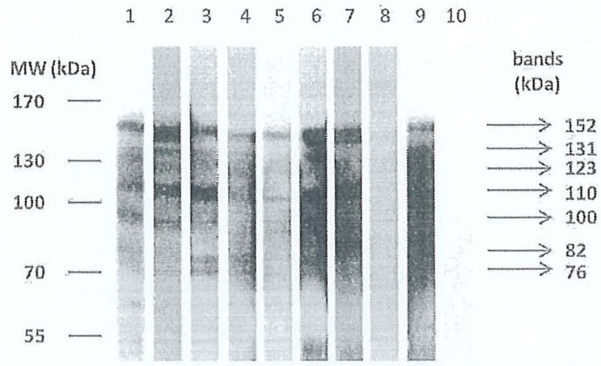
285 14. **Slamovits, C.H. and P.J. Keeling.** 2006. Pyruvate-phosphate dikinase of
286 oxymonads and parabasalia and the evolution of pyrophosphate-dependent
287 glycolysis in anaerobic eukaryotes. Eukaryot Cell. **5:**148-154.
288 15. **Stephen, P., et al.** 2008. Molecular modeling on pyruvate phosphate dikinase of
289 *Entamoeba histolytica* and in silico virtual screening for novel inhibitors. J.
290 Comput. Aided Mol. Des. **22:**647-660.
291 16. **Tanyuksel, M. and W.A. Petri, Jr.** 2003. Laboratory diagnosis of amebiasis.
292 Clin. Microbiol. Rev. **16:**713-729.
293 17. **Walsh, J.A.** 1986. Problems in recognition and diagnosis of amebiasis: estimation
294 of the global magnitude of morbidity and mortality. Rev. Infect. Dis. **8:**228-238.
295 18. **WHO, WHO/PAHO/UNESCO.** 1997. A consultation with experts on
296 amoebiasis. Mexico City, Mexico 28-29 January. Epidemiol. Bull. **18:**13-14.
297 19. **Wong, W.K., et al.** 2011. Comparison of protein-free defined media, and effect
298 of L: -cysteine and ascorbic acid supplementation on viability of axenic
299 *Entamoeba histolytica.* Parasitol. Res. **108**:425-30.
300 20. **Zambrano-Villa, S. et al.** 2002. How protozoan parasites evade the immune
301 response. Trends in Parasitology. **18:**272-278.
302 21. **Zeehaida, M., et al.** 2008. A study on the usefulness of Techlab *Entamoeba*
303 *histolytica* II antigen detection ELISA in the diagnosis of amoebic liver abscess
304 (ALA) at Hospital Universiti Sains Malaysia (HUSM), Kelantan, Malaysia. Trop.
305 Biomed. **25:**209-216.

306 22. **Zeehaida, M., et al.** 2009. Analysis of indirect hemagglutination assay results
307 among patients with amoebic liver abscess. International Medical Journal.
308 **16:**195-199.

309

310

1
2



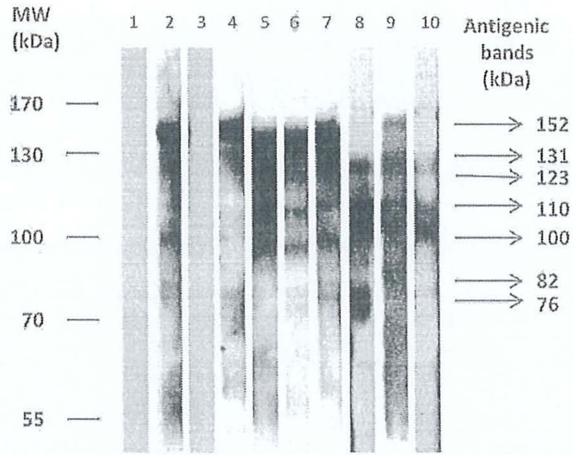
3

4 Figure 1 Representative IgG blot of ESA probed with human serum samples. Lanes
5 1-7: individual ALA serum samples from Group A; Lane 8: pooled IHA negative serum
6 sample (Group C); Lane 9: pooled ALA serum sample (positive control); Lane 10: TBS
7

1

2

3



4

5 Figure 2 Representative IgG blot of ESA when probed with human serum samples.

6 Lane 1: TBS; Lane 2: pooled ALA serum sample (positive control); Lane 3: pooled IHA

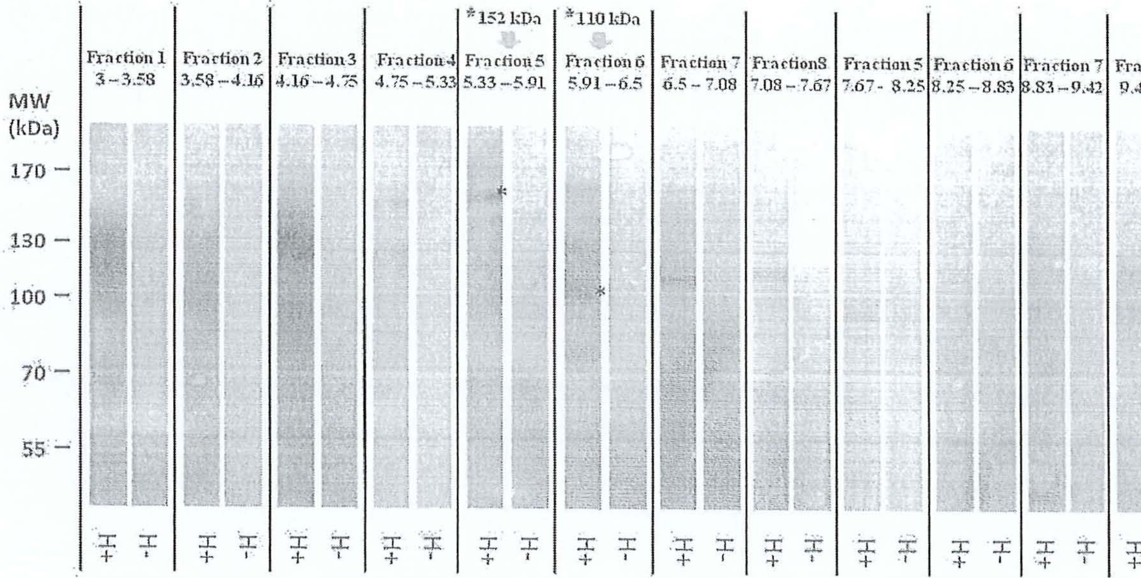
7 negative serum sample (Group D); Lanes 4-10: individual ALA serum samples from

8 Group B.

Table 1 Sensitivity and specificity of antigenic bands of ESA from IgG blots

Antigenic band, kDa	Human ALA serum samples, Group A, % [n=24]	Human ALA serum samples, Group B, % [n=14]	Sensitivity, % Mean (A,B) [n = 38]	Specificity, % Mean (C,D) [n = 63]
152	**79**	**93**	**84**	**100**
131	46	43	45	100
123	17	14	16	100
110	**79**	**86**	**82**	**100**
100	21	14	18	100
82	38	43	39	100
76	38	43	39	100

1



2

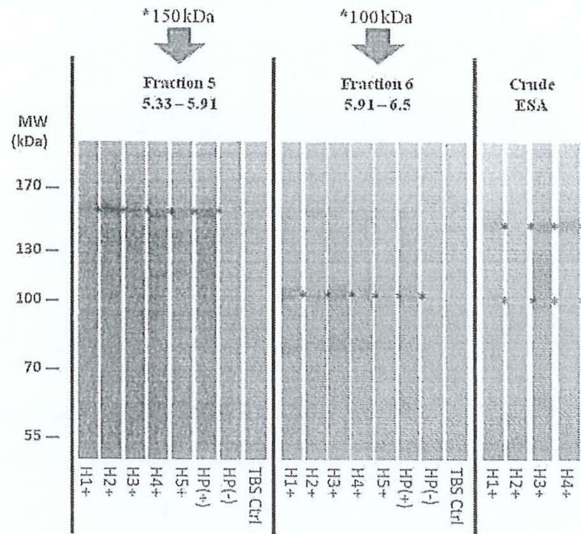
3 Figure 3 IgG blot of ESA separated by 2-DE, and probed with human serum

4 samples. H+: pooled positive ALA serum sample from Group B; H-: pooled IHA

5 negative serum sample (Group C).

6 (positive control); HP- : pooled IHA negative serum sample (group D).

1



2

3 Figure 4 IgG blot of ESA separated by 2-DE and probed with human serum

4 samples. H1+-H5+: individual ALA serum samples from Group B; HP+: pooled ALA

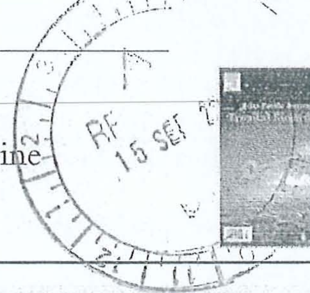
5 serum sample (positive control); HP- : pooled IHA negative serum sample (group D).

Asian Pacific Journal of Tropical Biomedicine (2012)61–65

Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage:www.elsevier.com/locate/apjtb



Document heading

Detection of *Entamoeba histolytica* in experimentally induced amoeb liver abscess: comparison of three staining methods

Tan Zi Ning[1], Wong Weng Kin[2], Shaymoli Mustafa[1], Arefuddin Ahmed[1], Rahmah Noordin[2], Tan Gim Cheon Olivos–Garcia Alfonso[4], Lim Boon Huat[1*]

[1]*School of Health Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia*
[2]*Institute for Research in Molecular Medicine, Universiti Sains Malaysia, 11800 Penang, Malaysia*
[3]*Department of Medical Microbiology and Parasitology, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia*
[4]*Departamento de Medicina Experimental, Facultad de Medicina, Universidad Nacional Autónoma de México, 04510 México D.F., México*

ARTICLE INFO

Article history:
Received 28 May 2011
Received in revised form 27 June 2011
Accepted 13 July 2011
Available online 28 January 2012

Keywords:
Entamoeba histolytica
Amoebic liver abscess
H&E
PAS
IHC
Trophozoite

ABSTRACT

Objective: To compare the efficacy of three different tissue stains, namely haematoxylin a eosin (H&E), periodic–acid Schiff (PAS) and immunohistochemical (IHC) stains for detect of *Entamoeba histolytica* (*E. histolytica*) trophozoites in abscessed liver tissues of hams **Methods:** Amoebic liver abscess was experimentally induced in a hamster by injecting 1 × of axenically cultured virulent *E. histolytica* trophozoites (HM1–IMSS strain) into the portal v After a week post–inoculation, the hamster was sacrificed and the liver tissue sections w stained with H&E, PAS and IHC stains to detect the amoebic trophozoite. **Results:** The th stains revealed tissue necrosis and amoebic trophozoites, but with varying clarity. H&E and stained the trophozoites pink and magenta, respectively, however it was difficult to differenti the stained trophozoites from the macrophages because of their similarity in size and morpholo On the other hand, IHC stain revealed distinct brown appearance of the trophozoites in infected liver tissues. **Conclusions:** It can be concluded that out of the three stains, IHC is best for identification of *E. histolytica* trophozoites in tissue sections.

1. Introduction

Amoebic liver abscess (ALA) is the most common clinical presentation of extraintestinal infection of the intestinal protozoon, *Entamoeba histolytica* (*E. histolytica*). This illness is prevalent worldwide and endemic in tropical countries such as India, Bangladesh, tropical African countries, some areas in Brazil and Mexico, China and South–east Asia. Although less than 1% of patients infected with *E. histolytica* develop ALA, this still represents an alarming number. The ailment is easily acquired in poor sanitation area, via ingestion of infective *E. histolytica* cysts present in contaminated hands, food or water. Interestingly, the incidence rate is also increasingly reported in no endemic and developed countries such as USA a European countries because of the ease of world travel a immigration of people from endemic areas[1–3].

Pathogenesis of ALA is known to be very complicat It develops through the hematological dissemination the pathogenic trophozoites into liver via the tributaries portal vein after invasion of colonic mucosa, resulting in formation of solitary or multiple abscesses regularly fou in the right liver lobe[4]. The common virulence fact involved include Gal/GalNAc specific lectin, cyste proteinases, amoebapores and lipophosphopeptidogly molecules[5,6]. In the formation of ALA, the gene sequence of morphological changes in liver tissues invol acute inflammation where the acute cellular infiltratio composed of polymorphonuclear leukocytes which surro the centrally located amoebas, then progress to granulo formation after the leukocytes were being replaced macrophages and epithelioid cells and subsequen

*Corresponding author: Lim Boon Huat, School of Health Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia.
Tel: (+6) 097677619
Fax: (+6) 097677515
E–mail: limbh493@gmail.com

Foundation Project: Supported by a grant from Universiti Sains Malaysia (grant No. 1001/PPSK/813009).

followed by extensive necrosis with fused granulomas[7–9].

Several kinds of laboratory animal models have reportedly been used to study the formation of ALA. Since 1950s, inoculation routes such as direct intrahepatic, intracaecal, intraperitoneal and intraportal were performed to induce ALA in hamster, mouse and gerbil. Currently, the intraportal injection of *E. histolytica* trophozoites in hamster has been widely used to produce ALA[9,10] and this technique is adopted in the present study.

A good staining method is pertinent in the pathogenesis study on ALA. An excellent stain facilitates visualization of the morphological changes in liver tissues and also differentiates the amoebas against surrounding cells such as hepatocytes, macrophages and other cell types[11]. The staining techniques reportedly used are haematoxylin and eosin (H&E), periodic–acid Schiff (PAS) and immunostaining. However, comparison of the efficiency of these staining methods in detecting amoebas has not been reported. Thus, this study was aimed to compare the efficacy of H&E, PAS and immunohistochemical (IHC) stains for detection of *E. histolytica* trophozoites in liver tissue of hamster with ALA.

2. Materials and methods

2.1. Development of ALA in experimentally induced hamster

ALA was induced in a Syrian golden hamster as described by Olivos–Garcia and Weber *et al*[12,13]. Briefly, 1×10^6 of axenically cultured virulent strain *E. histolytica* trophozoites (HM1–IMSS) was suspended in 0.2 mL phosphate buffer saline (PBS) and then inoculated into the portal vein of an anesthetized male hamster. After one week post–inoculation, the animal was sacrificed with a three–time overdose of pentobarbital. Immediately after the animal became unconscious, cardiac puncture was performed to collect the blood, then transferred into a sterile 1.5 mL microfuge tube and allowed to clot. The hamster serum containing polyclonal antibody against *E. histolytica* was then stored at −20 ℃ until used. The liver was removed aseptically, followed by fixation in 10% formalin. The same procedures were performed in the control healthy hamster, except that the injection fluid comprised 0.2 mL PBS. The animal experimentation was approved by USM Animal Research Ethics Committee [No. Animal Ethics Approval: USM/Animal Ethics Approval/2008/(40)(129)].

2.2. Tissue processing

Both infected and healthy formalin–fixed livers were cut into small pieces and kept in separate cassettes. The tissues were then processed overnight in an automated tissue processer (Leica TP 1020, Germany), which involved 1 h fixation, dehydration through graded alcohols for a total of 6 h, followed by 3 h clearing with xylene and 4 h tissue impregnation with embedding medium. The processed liver tissues were then embedded in paraffin wax to produce tissue blocks. Four μm thick formalin–fixed, paraffin–embedded tissue sections were cut with a microtome (Microm HM 325 Rotary Microtome, Germany) and subsequently stained with the three stains. Triplicate tissue sections were prepared for each stain.

2.3. Histochemical staining methods

2.3.1. H&E stain

Staining of the processed tissue sections was performed according to the standard protocol as described by Bancroft and Gamble with some modifications[14]. In brief, processed tissues were deparaffinized with two changes of xylene for 2 min each, rehydrated with two changes of absolute, 95% and 80% alcohols for 2 min each, followed by washing in running tap water for 5 min. Then, the tissues were stained with Harris's haematoxylin (Sigma–Aldrich, USA) for 20 min and washed in running tap water. Differentiation with 1% acid alcohol was carried out for 10 sec, followed by washing and bluing by dipping the tissues in ammonia water for 10 sec. After a washing step, the tissues were counterstained with eosin Y (Sigma–Aldrich, USA) for 2 min, dehydrated with increasing graded of alcohols for 2 min each, cleared with two changes of xylene for 2 min each and finally mounted with dibutyl phthalate xylene (DPX).

2.3.2. PAS stain

Slides were prepared based on the conventional protocol described by Bancroft and Gamble[14]. Briefly, processed tissues underwent the same deparaffinization, rehydration and washing steps as mentioned in the H&E stain. Next, the tissues were treated with periodic acid solution (Sigma–Aldrich, USA) for 5 min and washed with distilled water for 5 min. The tissues were then covered with Schiff's reagents for 10 min, followed by washing in running tap water for 5 min. Counterstaining was performed with Harris's haematoxylin (Sigma–Aldrich, USA) for 1 min, then washed in running tap water for 5 min and differentiated with 1% acid alcohol. Subsequently, the tissues were dipped in ammonia water for 10 sec until the sample turned blue, washed in running tap water for 5 min, followed by dehydration with increasing graded of alcohols, cleared with xylene and mounted with DPX.

2.4. Immunohistochemical staining method (IHC stain)

Indirect staining was performed on processed tissue sections with some modifications of the standard protocol as described by Bancroft and Gamble[14]. First, the tissues

were deparaffinized with two changes of xylene for 5 min each, followed by rehydration with two changes of absolute, 70% and 50% alcohols for 3 min each and washing in running tap water for 5 min. Tissues were then blocked with 3% hydrogen peroxide for 5 min, dipped in distilled water for 5 min and followed by 30 min incubation with 1:100 dilution of the corresponding polyclonal hamster serum sample *i.e.* sera from the ALA–induced hamster and control hamster used for the infected and control tissues, respectively. Washing steps were then carried out five times with PBS–Tween 20 (PBST), 2 min each. Tissues were incubated with 1:1 000 dilution of HRP–conjugated anti–hamster antibody (Sigma–Aldrich, USA) for 30 min and again washed with PBST. After washing, the tissues were developed with 3,3′–diaminobenzidine (DAB) substrate solution for 3 min and again washed with PBST. Finally, the tissues were counterstained with Harris's haematoxylin (Sigma–Aldrich, USA) for 1 min, followed by washing, differentiation with 1% acid alcohol, bluing with ammonia water, another washing step, dehydration with increasing graded alcohols, clearance with xylene and then mounted with DPX.

Finally, the three differently stained tissues were observed under a light microscope at different magnifications (40×, 100× and 400×) and the images were captured using image analysis system (Nikon eclipse 80i, Japan). Comparisons on the ease and clarity of *E. histolytica* trophozoites detection were then made based on the captured images.

3. Results

Gross examinations of both the infected and non–infected liver tissues were performed prior to processing for histology. The infected liver was found to be enlarged and studded with multiple small yellow–white abscesses, whereas the non–infected liver was normal in size with a smooth clean surface (Figure 1). All the triplicate stained tissue slides revealed similar overall appearance. The healthy liver tissue sections revealed intact hepatic lobules with central veins and cords of radiating hepatocytes surrounded by the portal triads. On the contrary, in sections from infected liver tissue, a well defined endothelial layer of central vein was not observed as seen in normal tissue section (Figure 2). The abscesses in the infected tissue were seen as foci of extensive necrolysis and degenerative changes. Efficacy of each staining method was compared in terms of the ease and clarity of trophozoites detection from tissue sections. With H&E stain, the trophozoites were stained pink whereas the PAS stain outlined the trophozoites magenta in colour. Both the stains could not differentiate the trophozoites clearly, as the amoebas resembled the macrophages. However, with the immunostain, the trophozoites were stained brown in colour, an end–product of the enzymatic reaction between DAB and horseradish peroxidase. Consequently, the appearances IHC–stained trophozoites were easily identified from t background of inflamed and necrotic tissues (Figure 3). Figure 4, the images captured from IHC stained slides clea revealed central necrotic region in liver tissue surround by scanty inflammatory cells with amoebic trophozoi along the margins. Islands of better preserved liver tiss were also seen scattered among the necrotic foci.

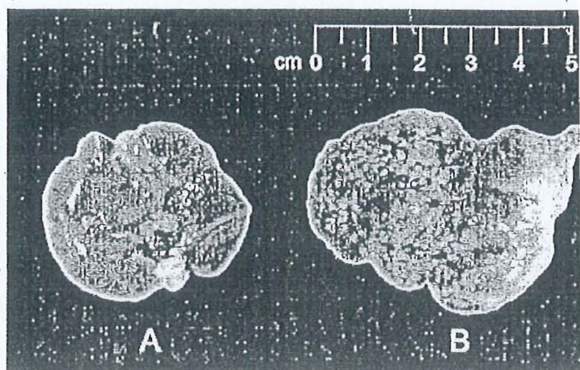


Figure 1. Gross appearance of hamster livers.
A: Non–infected healthy liver with a smooth and clean surface; B: One–week post inoculation abscessed liver with multiple tiny whitis spots.

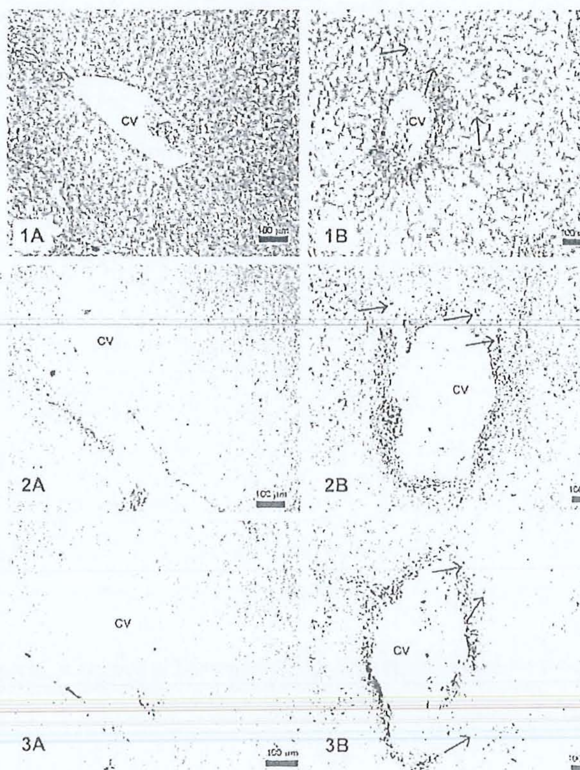


Figure 2. Photomicrographs showing normal liver tissues (left) and infected liver tissues (right) using three different staining techniques 1A, 1B: H&E stain; 2A, 2B: PAS stain; 3A, 3B: IHC stain (100×); CV: Central vein. *E. histolytica* trophozoites are indicated with arrows. A the sections from non–infected liver show normal liver architecture with intact central vein and cords of hepatocytes. Sections from the infected liver show necrolytic tissues with distorted central vein.

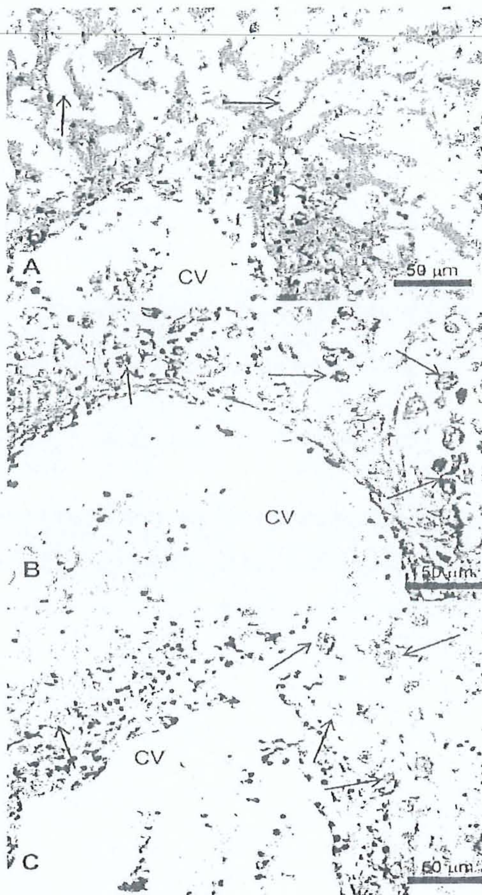


Figure 3. Micrographs indicating the different staining of *E. histolytica* trophozoites.
A: H&E stain; B: PAS stain; C: IHC stain (400×); CV: Central vein.
E. histolytica trophozoites are indicated with arrows. (A) Trophozoites (arrow) are visible as round, oval to pear shaped cells lying in lacunar spaces with occasional ingested red blood cells inside, very similar to macrophages in morphology. (B) PAS stained section showing the trophozoites (arrow) with magenta coloured cell membrane in a necrotic background. (C) IHC stain showing brown coloured trophozoites (arrow) with a distinct cell membrane easily identifiable against a background of necrosis and inflammation.

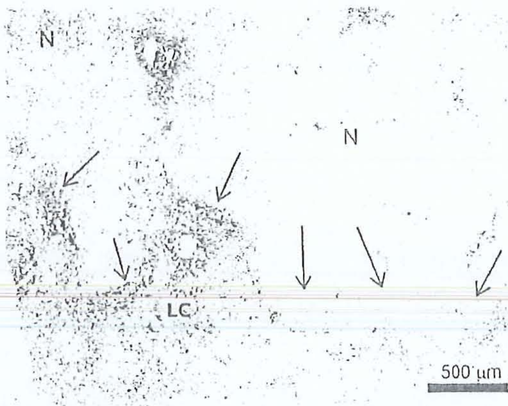


Figure 4. Photomicrograph from IHC stained liver tissue from infected hamster showing extensive necrosis representing coalescing microabscesses.
E. histolytica trophozoites (brownish, marked with arrow) are seen along the abscess margins invading the better preserved liver tissue. Magnification: 40×. N: necrotic area; LC: well–preserved liver cells.

4. Discussion

ALA has been known to be a potentially fatal extraintestinal infection of amoebiasis. Multiple factors involving parasite and the host have been reported to be involved in the development of ALA. The general concept of development of ALA involves the adaptation and survival of amoebas in liver tissue[9]. Rigothier *et al*[11], reported that there was massive death of parasites after a few hours of post–infection and inflammation in the hamster liver tissue was caused. After 12 h, the parasites started multiplying and the size of inflammation foci increased. In addition, other factors such as oxygen reduction ability, complement resistant, ROS and NOS scavenger capacity and immune evasion of the parasites also contribute to the parasites survival. Once the parasites are able to adapt to the environment in the liver, inflammation will be stimulated and followed by extensive tissue destruction[8,15,16].

In this study, the results showed that tissue destruction and amoebae in the tissue sections can be visualized by all the three stains, but with varying clarity. H&E and PAS stains required high technical expertise to identify and interpret the staining results. Even though H&E stain is the most widely employed histology stain to demonstrate the morphology of different cells and tissue[14], it has been reported to be not ideal for detection of amoebic trophozoites especially in the examination of fixed and stained biopsy samples due to the difficulty in differentiating the stained trophozoites from the surrounding tissues. PAS stains tissue carbohydrates magenta, and it is commonly used to stain liver glycogen[14]. The problem arises because *E. histolytica* trophozoites are also magenta in colour when stained with PAS, possibly due to the presence of glycoprotein in the amoeba cell membrane[17]. Thus, with both the H&E and PAS stains, amoebic trophozoites were difficult to differentiate from macrophages because of their similarities in size and morphology[18].

In comparison, IHC is presumed to be more specific as it is the consequence of specific reactions between antigens of amoebic trophozoite and antibodies against them. In this study, immunostaining gave more distinct and easily identifiable appearance of the trophozoites in a background of necrosis and inflammation as compared with the other two staining techniques. Even though numerous reported studies on amoebic pathogenesis utilized H&E and PAS stains, this study showed that IHC stain was more superior than the two stains. As was previously described for hamster and human ALA[9,19], the images captured from IHC stained slides clearly revealed central necrotic region in liver tissue surrounded by scanty inflammatory cells with amoebic trophozoites along the margins. Islands of better preserved liver tissue were also seen scattered among the necrotic foci. Moreover, serum sample could easily be obtained from 5–7 days post–infected hamster, and contained sufficient polyclonal antibodies that recognize *E. histolytica* trophozoites[20].

A previous study has reported that monoclonal antibody can be used in cryopreserved tissue section to stain amoeba but not in formalin–fixed, paraffin–embedded tissue[21]. However, this study showed that amoeba in paraffin–

embedded tissue can be visualized when polyclonal antibody was employed. The use of polyclonal antibody may be able to show stronger antigen recognition on amoebas in the formalin fixed tissue sections as compared with monoclonal antibodies which only recognize single epitopes. Also, processed tissue is favored to cryopreserve tissue because the structures of amoeba are physically supported by the embedding medium, while amoebic structure might be lost with frozen treatment due to the water crystallization.

Nowadays, in the diagnosis of amoebiasis, stool, blood, liver pus, urine and saliva samples are often investigated with various molecular-based and immunological-based techniques[22-25], whereas staining techniques are hardly reported. However, IHC is still relevant for confirmation of numerous pathogenic diseases[14], but rarely reported for use in the investigation of invasive amoebiasis. Thus, it is potentially important as a confirmatory test for ALA if sample from aspiration of liver abscess, liver biopsy or autopsy is available.

In conclusion, in this study, IHC stain was found to be more superior than H&E and PAS stains for detection of *E. histolytica* trophozoites in the infected tissues because the IHC allowed easy identification of brown-stained amoebas among the inflamed and necrotic liver cells.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

This study was funded by a research university grant from Universiti Sains Malaysia (1001/PPSK/813009). The first two authors received financial support through the USM Fellowship.

References

[1] Rao S, Solaymani-Mohammadi S, Petri WA, Parker SK. Hepatic amebiasis: a reminder of the complications. *Curr Opin Pediatr* 2009; **21**(1): 145–149.

[2] Parija SC. *Textbook of medical parasitology protozoology & helminthology.* 3rd ed. New Delhi: All India Publishers & Distributors; 2008.

[3] Seeto RK, Rockey DC. Amebic liver abscess: epidemiology, clinical features, and outcome. *West J Med* 1999; **170**(2): 104–109.

[4] Salles JM, Moraes LA, Salles MC. Hepatic amebiasis. *Braz J Infect Dis* 2003; 7(2): 96–110.

[5] Ackers JP, Mirelman D. Progress in research on *Entamoeba histolytica* pathogenesis. *Curr Opin Microbiol* 2006; 9(4): 367–373.

[6] Baxt LA, Singh U. New insights into *Entamoeba histolytica* pathogenesis. *Curr Opin Infect Dis* 2008; **21**(5): 489–494.

[7] Costa CA, Fonseca TH, Oliveira FM, Santos JF, Gomes MA, Caliari MV. Influence of inflammation on parasitism and area of experimental amoebic liver abscess: an immunohistochemical and morphometric study. *Parasit Vectors* 2011; 4(1): 27.

[8] Guo X, Houpt E, Petri WA. Crosstalk at the initial encounter: interplay between host defense and ameba survival strategies. *Curr Opin Immunol* 2007; **19**(4): 376–384.

[9] Santi-Rocca J, Rigothier MC, Guillen N. Host-micro interactions and defense mechanisms in the development amoebic liver abscesses. *Clin Microbiol Rev* 2009; **22**(1): 65–75.

[10] Tsutsumi V, Shibayama M. Experimental amebiasis: a select review of some *in vivo* models. *Arch Med Res* 2006; **37**(2): 210–22(

[11] Rigothier MC, Khun H, Tavares P, Cardona A, Huerre M, Guill N. Fate of *Entamoeba histolytica* during establishment amoebic liver abscess analyzed by quantitative radioimaging a histology. *Infect Immun* 2002; **70**(6): 3208–3215.

[12] Olivos-Garcia A, Nequiz-Avendano M, Tello E, Martinez R Gonzalez-Canto A, Lopez-Vancell R, et al. Inflammatio complement, ischemia and amoebic survival in acu experimental amoebic liver abscesses in hamsters. *Exp M Pathol* 2004; **77**(1): 66–71.

[13] Weber C, Blazquez S, Marion S, Ausseur C, Vats D, Krzemins M, et al. Bioinformatics and functional analysis of an *Entamoel histolytica* mannosyltransferase necessary for parasit complement resistance and hepatical infection. *PLoS Negl Tro Dis* 2008; 2(2): e165.

[14] Bancroft JD, Gamble M. *Theory and practice of histologic techniques.* 5th ed. China: Churchill Livingstone; 2002.

[15] Olivos-Garcia A, Saavedra E, Ramos-Martinez E, Nequiz M Perez-Tamayo R. Molecular nature of virulence in *Entamoeb histolytica. Infect Genet Evol* 2009; 9(6): 1033–1037.

[16] Wong-Baeza I, Alcantara-Hernandez M, Mancilla-Herrera Ramirez-Saldivar I, Arriaga-Pizano L, Ferat-Osorio E, et al. Th role of lipopeptidophosphoglycan in the immune response t *Entamoeba histolytica. J Biomed Biotechnol* 2010; **2010**: 254521.

[17] Aley SB, Scott WA, Cohn ZA. Plasma membrane of *Entamoeba histolytica. J Exp Med* 1980; **152**(2): 391–404.

[18] Kumar V, Abbas AK, Fausto N, Aster JC. *Robbins & Cotran pathologic basis of disease.* 8th ed. Philadelphia: Saunder Elsevier; 2010.

[19] Costa CA, Nunes AC, Ferreira AJ, Gomes MA, Caliari MV *Entamoeba histolytica* and *E. dispar* trophozoites in the liver o hamsters: *in vivo* binding of antibodies and complement. *Parasi Vectors* 2010; 3(1): 23.

[20] Compton SR, Riley LK. Detection of infectious agents in laborator rodents: traditional and molecular techniques. *Comp Med* 2001 **51**(2): 113–119.

[21] Sherchand JB, Thammapalerd N, Riganti M, Tharavani S, Punpoowong B. Monoclonal antibody-base immunohistochemical demonstration of *Entamoeba histolytic* in liver tissues of experimentally infected hamster (*Mesocricetu auratus*). *Int J Parasitol* 1994; 24(6): 909–916.

[22] Haque R, Kabir M, Noor Z, Rahman SM, Mondal D, Alam F, et al Diagnosis of amebic liver abscess and amebic colitis by detectio of *Entamoeba histolytica* DNA in blood, urine, and saliva by real-time PCR assay. *J Clin Microbiol* 2010; 48(8): 2798–2801.

[23] Khairnar K, Parija SC. Detection of *Entamoeba histolytica* DN in the saliva of amoebic liver abscess patients who received prio treatment with metronidazole. *J Health Popul Nutr* 2008; **26**(4 418–425.

[24] Parija SC, Khairnar K. Detection of excretory *Entamoeb histolytica* DNA in the urine, and detection of *E. histolytica* DN and lectin antigen in the liver abscess pus for the diagnosis o amoebic liver abscess. *BMC Microbiol* 2007; 7: 41.

[25] Othman N, Mohamed Z, Verweij JJ, Huat LB, Olivos-Garcia A Yeng C, et al. Application of real-time polymerase chain reactio in detection of *Entamoeba histolytica* in pus aspirates of live abscess patients. *Foodborne Pathog Dis* 2010; 7(6): 637–641.

Dear Dr. Lim:

Here is a copy of the decision letter for manuscript "Analysis of Entamoeba histolytica excretory-secretory antigen (ESA) and identification of a new potential diagnostic marker" by Weng Kin Wong, Zi Ning Tan, Nurulhasanah Othman, Boon Huat Lim, Zeehaida Mohamed, Alfonso Olivos Garcia, and Rahmah Noordin (CVI05356-11R1), for which you were a contributing author.

Sincerely,

W. Ray Waters
Editor
Clinical and Vaccine Immunology

Subject: CVI05356-11R1 Decision Letter

Prof. Rahmah Noordin
Universiti Sains Malaysua
Penang
Malaysia

Re: CVI05356-11R1 (Analysis of Entamoeba histolytica excretory-secretory antigen (ESA) and identification of a new potential diagnostic marker)

Dear Prof. Rahmah Noordin:

Your manuscript has been accepted, and I am forwarding it to the ASM Journals Department for publication. For your reference, ASM Journals' address is given below. Before it can be scheduled for publication, your manuscript must be checked by the ASM production editor to make sure that all elements meet the technical requirements for publication. Diane Smith, the production editor for Clinical and Vaccine Immunology, will contact you if anything needs to be revised before copyediting and production can begin. Otherwise, you will be notified when your proofs are ready to be viewed.

Corresponding authors who are ASM members are entitled to discounted page charges, reprint fees, and color figure fees. For 2011 issues, page charges (subject to change without notice) will be assessed at $67 per printed page for the first eight pages and $125 for each page in excess of eight for a corresponding author who is an ASM member or $80 per printed page for the first eight pages and $250 for each page in excess of eight for a nonmember corresponding author. A corresponding author who is not a member may join ASM to obtain the member rate. If the research was not supported, you may send a

request for a waiver of page charges to the Director, Journals. Waivers apply only to page charges. Responsibility for color charges and other publication fees remains with the author. For more details, including types of articles not charged, see the Instructions to Authors. Color charges for 2011 issues are $45 per figure for a member corresponding author and $75 per
figure for a nonmember corresponding author. Please note that any figures you supplied in color will automatically be processed as color and you will be responsible for color costs.

To offset the costs associated with posting journal article supplemental material, ASM charges a flat fee for authors who wish to post supplemental material as an adjunct to their published article. The 2011 fee is $190, with a limit of 10 supplemental files per article. (Exceptions: Minireviews and Commentaries are exempt from this fee.)

ASM is also now offering authors the option of paying a fee to allow immediate open access to both the preliminary "Accepts" version and the final, typeset version of their articles. The 2011 fee is $2,000, which is in addition to current publication charges. The open access provided through NIH's PubMed Central repository is separate and will continue regardless; all primary research published in ASM journals is freely available through PubMed Central 6 months after publication. Please contact the ASM production editor immediately if you wish to pay the optional open access fee.

Note the following about publish ahead of print: For its primary-research journals, ASM posts online PDF versions of manuscripts that have been peer reviewed and accepted but not yet copyedited. This feature is called "CVI Accepts" and is accessible from the Journals website. The manuscripts are published online as soon as possible after acceptance, on a weekly basis, before the copyedited, typeset versions are published. They are posted "As Is" (i.e., as submitted by the authors at the modification stage), and corrections/changes are NOT accepted. Accordingly, there may be differences between the CVI Accepts version and the final, typeset version. The manuscripts remain listed on the CVI Accepts page until the final, typeset versions are posted, at which point they are removed from the CVI Accepts page. They are under subscription access control until 6 months after the typeset versions are posted, when access to all forms becomes free to everyone. Any supplemental
material intended, and accepted, for publication is not posted until publication of the final, typeset article.

IMPORTANT NOTICE: Effective April 2008, the NIH initiated a policy requiring that all investigators funded by the NIH either submit or have submitted for them to the National Library of Medicine's PubMed Central an electronic version of their final peer-reviewed manuscripts upon acceptance for publication, to be made publicly available no later than 12 months after the official date of publication. Authors of ASM journal articles are automatically in compliance with this policy and need take no action themselves. For the past several years, the ASM has deposited in PubMed Central all publications from all ASM journals. Further, all primary ASM literature is made available to everyone, free, 6 months after publication through PubMed Central,

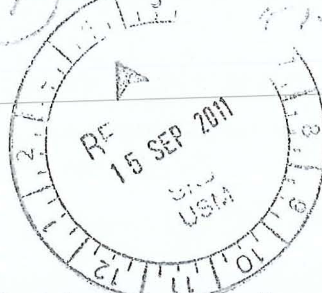
HighWire, and international PubMed Central-like repositories. By having initiated these policies, the ASM is in full compliance with NIH policy.

Thank you for submitting your paper to CVI.

Sincerely,
W. Ray Waters
Editor, Clinical and Vaccine Immunology

Journals Department
American Society for Microbiology
1752 N St., NW
Washington, DC 20036
E-mail: dsmith@asmusa.org
Phone: 1-202-942-9288
Fax: 1-202-942-9355

Tropical Biomedicine 27(1): 79–88 (2010)



Identification of *Entamoeba histolytica* trophozoites in fresh stool sample: comparison of three staining techniques and study on the viability period of the trophozoites

Tan, Z.N.[1], Wong, W.K.[3], Nik Zairi, Z.[2], Abdullah, B.[2], Rahmah, N.[3], Zeehaida, M.[2], Rumaizi, S.[4], Lalitha, P.[5], Tan, G.C.[2], Olivos-Garcia, A.[6] & Lim, B.H.[1]
[1] School of Health Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia.
[2] Department of Medical Microbiology and Parasitology, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia
[3] Institute for Research in Molecular Medicine, Universiti Sains Malaysia, 11800, Penang, Malaysia.
[4] Laboratory Animal Research Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia.
[5] Faculty of Applied Sciences, AIMST University, 08000 Sungai Petani, Kedah, Malaysia.
[6] Departamento de Medicina Experimental, Facultad de Medicina, Universidad Nacional Autónoma de México, 04510 México D.F., México
Corresponding author e-mail: limbh493@gmail.com
Received 16 December 2009; received in revised form 13 January 2010; accepted 15 January 2010

Abstract. *Entamoeba histolytica* causes about 50 million infections worldwide with a death rate of over 100,000 annually. In endemic developing countries where resources are limited, microscopic examinations based on Wheatley trichrome staining is commonly used for diagnosis of intestinal amoebiasis. Other than being a time-consuming method, it must be performed promptly after stool collection as trophozoites disintegrate rapidly in faeces. The aim of this study was to compare the efficacies of Eosin-Y, Wheatley trichrome and Iodine stains in delineating the diagnostic features of the parasite, and subsequently to determine the suitable microscopy observation period for detection of erythrophagocytic and non-erythrophagocytic trophozoites spiked in semi-solid stool sample. Wheatley trichrome staining technique was performed using the standard method while the other two techniques were performed on the slides by mixing the respective staining solution with the spiked stool sample. One million of axenically cultured non-erythrophagocytic *E. histolytica* and erythrophagocytic *E. histolytica* were separately spiked into 2 g of fresh semi-solid faeces. Percentage viability of the trophozoites in the spiked stool sample was determined at 30 minute intervals for eight hours using the 0.4% Trypan blue exclusion method. The results showed that Eosin-Y and Wheatley trichrome stained the karyosome and chromatin granules better as compared to Iodine stain. The percentage viability of non-erythrophagocytic trophozoites decreased faster than the erythrophagocytic form in the first 5 hours and both dropped to ~10% in the 6th hour spiked sample. In conclusion, Eosin-Y staining technique was found to be the easiest to perform, most rapid and as accurate as the commonly used Wheatley trichrome technique; Eosin-Y stained slide sealed with DPX could also be kept as a permanent record. A period not exceeding 6 hours after stool collection was found to be the most suitable in order to obtain good microscopy results of viable trophozoites.

INTRODUCTION

Entamoeba histolytica is an enteric anaerobic protozoan parasite that causes about 50 million infections with a death rate of over 100 000 worldwide annually (WHO, 1997; Jackson, 1998; Zlobl, 2001; Fotedar *et al.*, 2007). The amoebic infection is the third most common cause of death among parasitic diseases, after malaria and schistosomiasis (Tanyuksel & Petri, 2003). The disease is widely reported in developing countries like India and Bangladesh, tropical African countries and in some areas in Brazil and Mexico. The incidence is increasing in non-endemic and developed countries such

as the USA and European countries, due to the ease of world travel and immigration of people from endemic areas (Nari *et al.*, 2008). High risk people are those who travel to crowded endemic areas with low standards of hygiene and sanitation; and those who practice unnatural sexual activities such as direct anal-genital and/or oral-anal sex (Espinosa-Cantellano & Martinez-Palomo, 2000; Haque *et al.*, 2000; Zlobl, 2001; Fotedar *et al.*, 2007).

Entamoeba histolytica has a simple life cycle, in which the transmission is via the faecal-oral route. Infection occurs through ingestion of infective cysts (size 8-20 µm) or invasion of motile trophozoites (size 20-40 µm) (Martinez-Palomo, 1982; Lucas & Upcroft, 2001). The infection causes a variety of clinical presentations, from asymptomatic colonization to invasive amoebic dysentery and extraintestinal amoebiasis. Most infected individuals do not show clinical signs, and the problem is compounded by the lack of reliable and practical diagnostic tools (Martinez-Palomo, 1982; Huston *et al.*, 1999; Zlobl, 2001; Blessmann *et al.*, 2003; Huston, 2004; Fotedar *et al.*, 2007).

The routine diagnosis of amoebic dysentery is still based on identification of erythrophagocytic trophozoites in dysenteric specimens (Cheesbrough, 2005). This low- cost diagnostic technique is still the preferred method in developing countries although numerous molecular-based methods such as polymerase chain reaction and immunological-based methods such as enzyme-linked immunosorbent assay, have been reported to be effective in species-specific diagnosis of *E. histolytica* (Huston *et al.*, 1999; Tanyuksel & Petri, 2003; Visser *et al.*, 2006; Fotedar *et al.*, 2007). A major setback in microscopy is the requirement of freshly collected stool samples as the trophozoites had been reported to disintegrate in faeces from 30 minutes to 3 hours after collection (Gardner *et al.*, 1980; Tanyuksel & Petri, 2003; Fotedar *et al.*, 2007); nevertheless there is no conclusive published data to support this claim. Another disadvantage of microscopy is the time consuming Wheatley trichrome staining process, which requires at least 42 minutes to perform (Flournoy *et al.*, 1982).

As amoebiasis mostly occurs in resource-tight developing countries, microscopy technique will still remain the diagnostic method of choice. Laboratories worldwide reportedly used numerous successful staining methods such as Wheatley's trichrome, Iron hematoxylin, Giemsa, Wright's, Methylene blue, Chorazole Black E and Iodine-trichrome stains (Koontz & Weinstock, 1996; Tanyuksel & Petri, 2003; Fotedar *et al.*, 2007), but all are tedious and time-consuming. Hence, a simple, rapid and reliable staining technique is urgently needed. The objectives of this study were to compare the efficacies of Eosin-Y, Wheatley trichrome and Iodine in staining the characteristic features of the parasite; and subsequently to determine the most suitable microscopy observation period for detection of erythrophagocytic and non-erythrophagocytic trophozoites spiked in semi-solid stool sample.

MATERIALS AND METHODS

Staining of trophozoites

Approximately one million *E. histolytica* axenically cultured in TYI-S-33 medium was washed with 1X Phosphate Buffered Saline (PBS) and spiked in 2 g of fresh semi-solid stool sample obtained from a healthy volunteer. Then, spiked stool samples were stained separately with Wheatley trichrome, Iodine and Eosin-Y solution alcoholic with phloxine B (Sigma HT110316, USA) (Eosin-Y). Duplicate slide smears were prepared for each staining technique. Wheatley trichrome staining technique was performed based on the standard operating protocol (SOP) used at the Department of Medical Microbiology and Parasitology, School of Medical Sciences, Universiti Sains Malaysia, Malaysia. An applicator stick was used to smear ~2 mg of stool sample on a clean slide. The smeared slide was then immersed in Schauddin's fixative for 2 hours. This was followed by soaking the slide in succession in tincture of iodine, 70% alcohol, Wheatley

trichrome stain (REMEL Inc., Lenexa, USA), acid alcohol, absolute alcohol and xylene. Finally, the slide was mounted with dibutyl phthalate xylene (DPX) and observed under a light microscope at 1000X magnification. The iodine stained smeared slide was prepared based on the protocol suggested by Koontz & Weinstock (1996). Briefly, an applicator stick was used to mix ~2 mg of stool sample with ~30 µL Lugol's Iodine solution on a clean slide. A cover slip was placed on the sample and sealed with DPX, then observed under a microscope. In Eosin-Y staining technique, an applicator stick was used to mix ~30 µL of Eosin-Y with ~2 mg of stool sample on a clean slide. A cover slip placed over the sample was sealed with DPX, then observed under a microscope at 400X and 1000X magnification. The images of the trophozoites stained by all three methods were captured using an Olympus Image Analysis System (Olympus System Microscope Model BX41, Japan). Comparisons were made among the images of the three types of stained trophozoites based on the clarity of their characteristic nuclear features.

Viability of non-erythrophagocytic and erythrophagocytic trophozoites in stool sample

About one million cultured trophozoites washed with 1X PBS were spiked into 2 g of fresh semi-solid stool sample. About 2 mg of the sample was mixed with 50 µl Trypan (0.4%) blue and the mixture was loaded into a Neubauer chamber to determine the viability of non-erythrophagocytic trophozoites by microscopy. The procedure was performed in duplicate and repeated every 30 minute intervals for eight hours.

In order to determine the viability of erythrophagocytic trophozoites, about 10 µL of blood was first added into a sterile microfuge tube containing 1×10^6 axenically cultured trophozoites. After 30 minutes, the trophozoites were washed with 1X PBS and spiked into 2 g of fresh semi-solid stool sample. Then, ~2 mg spiked stool sample was mixed with 0.4% Trypan blue, and the percentage viability was determined as described earlier.

Direct wet mounts were also prepared to observe the movement of motile trophozoites and their disintegration over time. An applicator stick was used to mix ~2 mg of spiked stool sample with ~30 µL normal saline (0.85% NaCl) on a clean slide. Then, a cover slip was placed on the sample and observed immediately under a light microscope.

RESULTS AND DISCUSSION

Staining of trophozoites

Images of the trophozoites were compared based on the detection of the characteristic features of trophozoites such as the chromatin granules that line the nuclear membrane and the small spherical karyosome at the centre of the nucleus. Nucleus of trophozoite has no fixed position in the cytoplasm, but moves freely and sometimes rotates rapidly (Martinez-Palomo, 1982). Thus, observation of the characteristic features of live trophozoites requires fine focusing of the optical microscope at 400X or 1000X magnification.

Permanent stains were much more effective than the direct wet mount for detection of trophozoites and/or cysts in stool specimens (Gardner *et al.*, 1980). Figure 1(a) shows the image of a Wheatley trichrome stained trophozoite; it was stained blue-purple with greenish background, with good delineation of the chromatin granules and karyosome. The stain provided a good contrast between the trophozoite and the background debris. However, an obvious disadvantage was the tedious protocol which required 2 hours fixation period and a total time of ~3 hours to complete. Appropriate fixation periods coupled with sufficient washing steps are pertinent in obtaining a well-stained nucleus, thus may require the preparation of a number of slides for each stool sample. Repeated use of acid alcohol in destaining trichrome stain will reduce its efficiency and subsequently require a longer destaining time although a better alternative is to use a fresh solution. The suggested fixation time with Schauddin's fixative is between 2 to 24 hours. Any increase in

fixation time must be followed by an appropriate increase in washing time using tincture of iodine. Moreover, Schauddin's fixative, which killed and fixed the trophozoites contains mercury compound which is not environmentally-friendly (Garcia & Shimizu, 1998; Amin, 2000). This staining technique demands technical skills of an experienced microscopist, and would be daunting to those unskilled personnel who have to perform the technique occasionally.

Iodine stain is mostly used to identify *E. histolytica* cysts in stool microscopic detection (Cheesbrough, 2005). However, Koontz & Weinstock (1996) reported that the stain could be used to delineate intestinal amoebas by negating the motility of the trophozoites. As shown in figure 1 (b), the nuclear chromatin granules were only faintly stained and the karyosome remained unstained.

Figures 1(c) and 1(d) show Eosin-Y stained non-erythrophagocytic and erythrophagocytic trophozoites, respectively. The former shows a trophozoite with its well-stained nuclear chromatin granules and karyosome; and the latter reveals well-stained characteristic features of the erythrophagocytic amoebic trophozoite and the engulfed erythrocytes. The whole trophozoite was stained light red, and both the chromatin granules and karyosome showed distinctly dark appearances. Eosin-Y also clearly stained the engulfed erythrocytes.

Various types of eosin stains are available commercially and some are used as counterstain to haematoxylin in Haematoxylin and Eosin (H&E) stain. Its acidic property stains the basic components of a cell, such as cytoplasm, light red in colour. Others used eosin as an exclusion dye to stain dead trophozoites light red in colour to distinguish them from the unstained viable trophozoites (Mirelman *et al.*, 1987; Behnia *et al.*, 2008). The stain was also reportedly used to facilitate the detection of motile trophozoites by staining the background pink without staining the live parasites (Cheesbrough, 2005). Interestingly, phloxine B in Eosin-Y was reportedly used to stain nuclear structures in histological sections (SPI.Supplies, 2009). Until now, there were no reports on the use of phloxine B to stain nucleus of *E. histolytica*. Here, we showed that Eosin-Y was just as accurate as Wheatley trichrome staining method in identification of trophozoites in stool samples. Besides staining the characteristic nuclear features of the trophozoites and/or the engulfed erythrocytes, it could be performed easily to give spontaneous results. The Eosin-Y used in this study is commercially available in its working dilution, thus can be applied directly onto the stool samples without fuss. Alternatively, Eosin-Y staining solution can also be prepared by mixing 1% (w/v) Eosin-Y, 1% (w/v) phloxine B, 95% ethanol and glacial acetic acid in appropriate volumes (Mayer's, 2009).

Another major advantage of Eosin-Y staining technique is that the stained trophozoites could easily be visualized under 400X magnification. At this magnification, it is very difficult to identify the Wheatley trichrome stained trophozoites. The rounded shape and immotile trophozoites left for 3 hours in stool sample were also easily stained by Eosin-Y [Figure 1(e)]. In fact, this stain was able to preserve the general morphology of the trophozoite for more than 24 hours. This was probably due to the presence of alcohol and glacial acetic acid in the stain, as these two chemicals are also used in Schauddin's fixative.

A major advantage of Wheatley trichrome staining technique is that it be used to prepare a permanent record of the stained amoebas. In contrast, the stained nuclear chromatin granules and karyosome of an Eosin-Y stained trophozoite gradually became fainter over time and almost indistinguishable from its cytoplasm after an hour [Figure 1(f)]. However, by sealing the edges of the cover slip to the slide with DPX, it prevented Eosin-Y from drying. This permanent record of the Eosin-Y stained slide could be stored longer if placed in a horizontal position (instead of a vertical position).

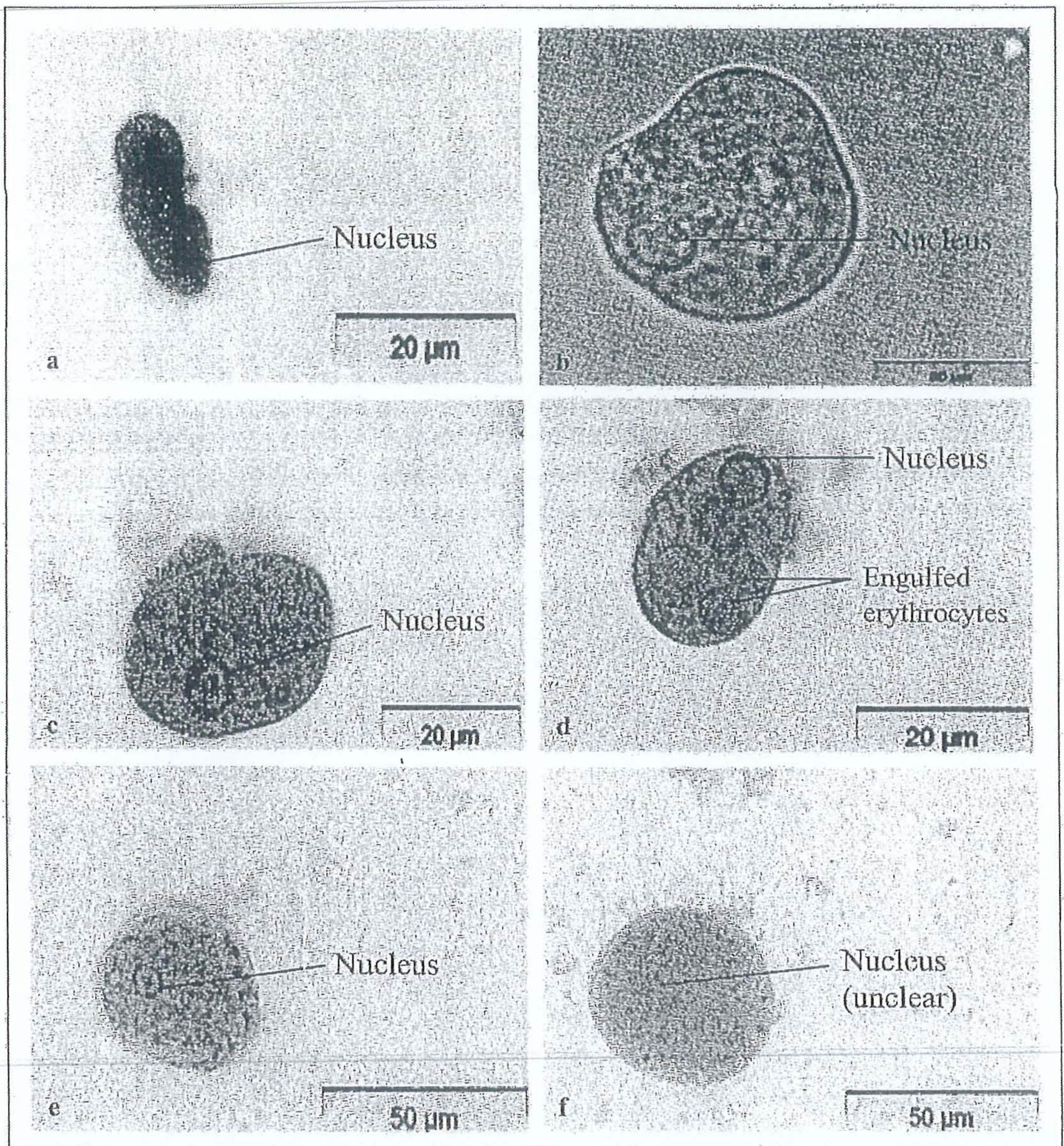


Figure 1. Stained trophozoites. (a) Wheatley trichrome stained trophozoite, 1000X magnification (b) Iodine stained trophozoite, 1000X magnification (c) Eosin-Y stained trophozoite, 1000X magnification (d) Eosin-Y stained erythrophagocytic trophozoite, 1000X magnification (e) Eosin-Y stained trophozoite showed clear chromatin granules and karyosome, 400X magnification (f) Eosin-Y stained trophozoite without DPX seal indicated unclear nuclear characteristics after an hour, 400X magnification.

Viability of non-erythrophagocytic and erythrophagocytic *E. histolytica* trophozoites in stool sample

Gonzalez-Ruiz *et al.* (1994) reported that trophozoites started to disintegrate rapidly as soon as they were in the faeces. However, the viability period of trophozoites outside its host was not studied. In the present study, the viability of trophozoites in stool sample was assessed by Trypan blue dye exclusion test whereby the dead trophozoites were stained blue and the live ones remained unstained (Figure 2). The viability chart of the non-erythrophagocytic trophozoites in spiked semi-solid stool is shown in (Figure 3). During the first hour, the percentage viability dropped rapidly and fluctuated at approximately 55%. This was probably due to the unfavourable conditions in the stool as compared to the optimal axenic

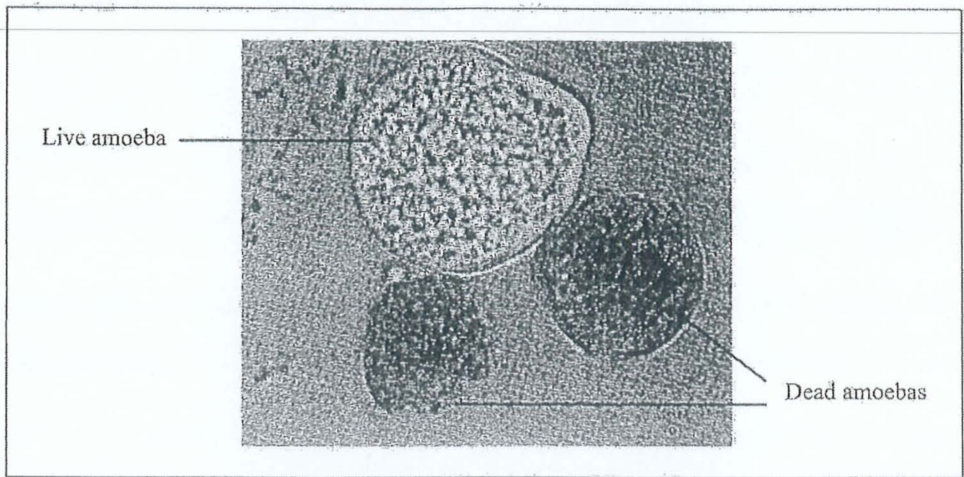


Figure 2. Trypan blue dye exclusion stained trophozoites, 1000X magnification.

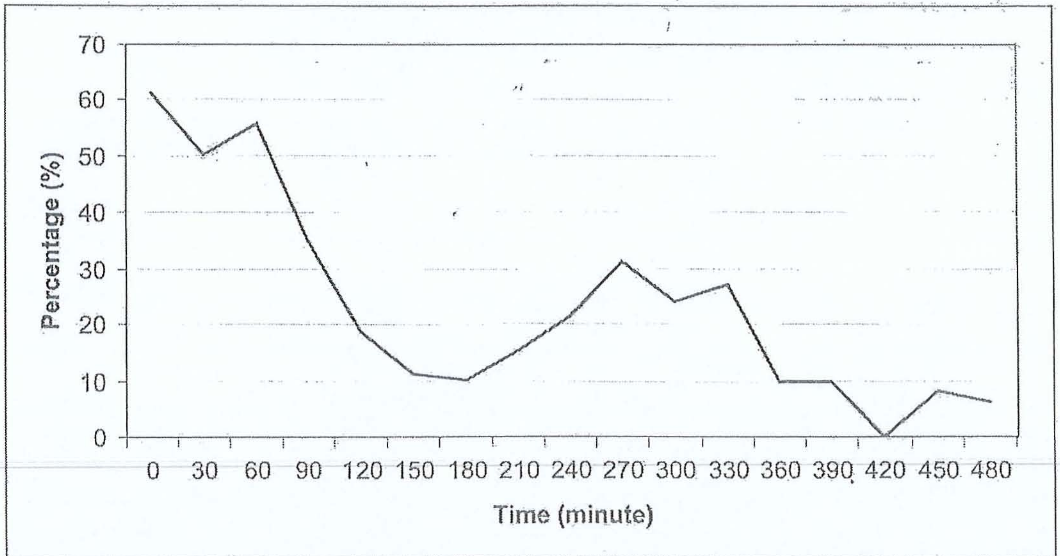


Figure 3. Viability chart of *E. histolytica* trophozoites in spiked semi-solid stool.

conditions of the trophozoites in TYI-S-33 medium at 36°C. Thereafter, the viability dropped to ~10% at the third hour. However, during the 3 to 5½ hours period, the percentage viability increased slightly, and then fluctuated around ~30%. At the 7th hour, none of the trophozoites was detected but ~10% viability was again observed at the 8th hour. This was probably due to the fact that *E. histolytica* in the stool samples was being challenged with a toxic high oxygen environment (30%) since it has been reported that amoebas can be supported in only less than 5% O_2 (Band & Cirrito, 1979). Figure 4 shows the viability chart of the erythrophagocytic trophozoites in spiked semi-solid stool. In comparison with figure 3, the percentages viability of erythrophagocytic trophozoites was higher (65% and 95%) during the first and third hours in stool sample. This was probably due to the antioxidant molecules (superoxide dismutase, catalase, glutathione, peroxirredoxin and vitamin E) present in the

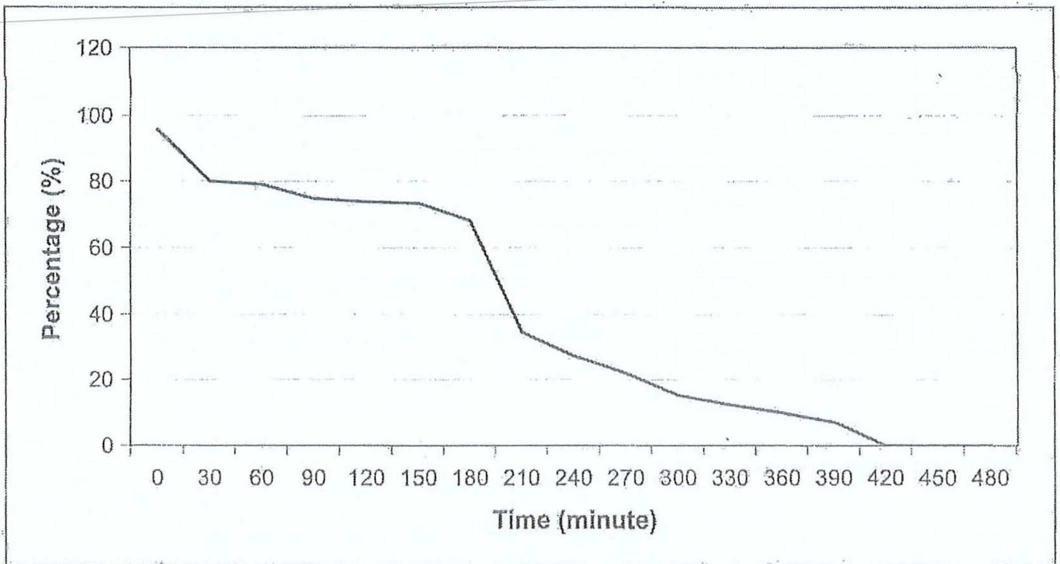


Figure 4. Viability chart of *E. histolytica* erythrophagocytic trophozoites in spiked semi-solid stool.

engulfed erythrocytes (Kuypers, 2007), which helped the amoeba to detoxify the reactive oxygen species generated during the oxygen reduction and/or because the erythrocytes were source of nutrients for the amoebas. Between the third and sixth hours, the mortality of the trophozoites increased gradually from about 65% to 90%, and none was detected from the seventh hour onwards. In general, the percentages viability of both forms of trophozoites dropped to ~10% at the sixth hour in semi-solid stool sample and none was detected from the seventh hour onwards. Since ~90% of the trophozoites were undetected at the sixth hour, microscopy detection to detect the amoebas should thus be performed within six hours after stool collection.

Observation of the direct wet mount slide preparation during the first hour in fresh semi-solid stool sample revealed that the trophozoites did not have fixed shape and were actively pushing out the ectoplasm to form pseudopodia, followed by the in-flowing endoplasm. In addition, the technique allowed the disintegration process of trophozoites to be observed over time (Figure 5a). Uroid of the amoeba was located at the posterior end of the live trophozoites. Faint engulfed erythrocytes were also visible but the characteristic nucleus was impossible to visualize without staining (Figure 5b). Thus the direct wet mount technique is neither sensitive nor reliable for detection of *E. histolytica* in stool samples.

All microscopy staining techniques (include those used in this study) cannot differentiate *E. histolytica* from the non-pathogenic *E. dispar*. However parasite identification by staining is still commonly used in developing endemic countries where resource are limited, as the costs of commercially available *E. histolytica* antigen detection tests are prohibitive. An important supportive evidence for microscopy is the detection of erythrophagocytic trophozoites in stool sample, although some non-pathogenic *Entamoeba* species may also ingest erythrocytes (Gonzalez-Ruiz *et al.*, 1994). Indiscriminate use of antiparasitic drugs may lead to development of drug-resistant. Thus, treatment should only be given to patients where the presence of *E. histolytica* in stool is confirmed, and no treatment should be administered if only *E. dispar* is found (WHO, 1997). Until now, light microscopic differentiation between the two amoeba species is not yet available and WHO has highlighted the urgent need in developing improved methods for the species-specific diagnosis of *E. histolytica* infection (WHO,

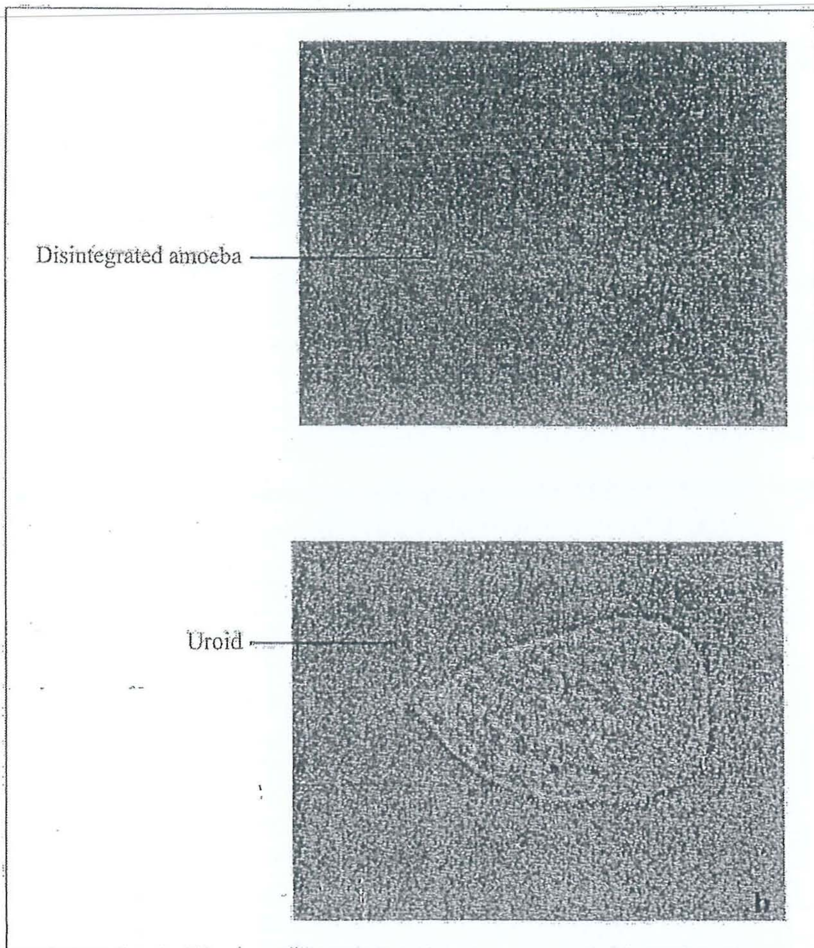


Figure 5. (a) Disintegrated trophozoite, 1000X magnification (b) Motile trophozoite with uroid at posterior end examined using direct wet mount, 1000X magnification.

1997). Hence efforts should also be on the search for stains which can specifically demonstrate structural compounds found in *E. histolytica* but absent in *E. dispar*.

In conclusion, this study showed that for microscopic identification of *E. histolytica* in patients' samples, Eosin-Y could stain the characteristic nuclear chromatin granules and karyosome of the trophozoites as accurately as the Wheatley trichrome, and better than the Iodine stain. Eosin-Y stained slide could also be kept as permanent record if the cover slip is sealed to the slide with DPX, however further studies are needed to determine the period of time before drying occurs. Nevertheless, Eosin-Y technique offers the added advantages of being rapid and easy to perform, thus is very useful for the purpose of identification of *E. histolytica* in patients' stool samples, especially in busy, and/or understaffed laboratories. The identification of *E. histolytica* was supported by the signs and symptoms presented by the patients and the detection of erythrophagocytic trophozoites. This study also suggests that the microscopy observation for viable trophozoites is best performed within the first 6 hours after stool collection.

Acknowledgement. This study was funded by a research university grant from Universiti Sains Malaysia (1001/PPSK/813009). The first author received financial support through

the university fellowship. Special thanks to Mohd Khairul Afif Azman for his technical assistance and Zainul Fadziruddin Zainuddin for proofreading the manuscript.

REFERENCES

Amin, O.M. (2000). Evaluation of a new system for the fixation, concentration, and staining of intestinal parasites in fecal specimens, with critical observations on the trichrome stain. *Journal of Microbiological Methods* **39**: 127-132.

Band, R.N. &, Cirrito, H. (1979). Growth response of axenic *Entamoeba histolytica* to hydrogen, carbon dioxide, and oxygen. *Journal Protozoology* **2**: 282-286.

Behnia, M., Haghighi, A., Komeylizadeh, H., Tabaei, S.J. & Abadi, A. (2008). Inhibitory effects of Iranian Thymus vulgaris extracts on *in vitro* growth of *Entamoeba histolytica*. *Korean Journal of Parasitology* **46**: 153-156.

Blessmann, J., Ali, I.K., Nu, P.A., Dinh, B.T., Viet, T.Q., Van, A.L., Clark, C.G. & Tannich, E. (2003). Longitudinal study of intestinal *Entamoeba histolytica* infections in asymptomatic adult carriers. *Journal of Clinical Microbiology* **41**: 4745-4750.

Cheesbrough, M. (2005). *District Laboratory Practice in Tropical Countries*. 2nd Edition. Cambridge University Press. pp. 200-202.

Espinosa-Cantellano, M. & Martinez-Palomo, A. (2000). Pathogenesis of intestinal amebiasis: from molecules to disease. *Clinical Microbiology Reviews* **13**: 318-31.

Flournoy, D.J., McNabb, S.J., Dodd, E.D. & Shaffer, M.H. (1982). Rapid trichrome stain. *Journal of Clinical Microbiology* **16**: 573-574.

Fotedar, R., Stark, D., Beebe, N., Marriott, D., Ellis, J. & Harkness, J. (2007). Laboratory diagnostic techniques for *Entamoeba* species. *Clinical Microbiology Reviews* **20**: 511-532.

Garcia, L.S. & Shimizu, R.Y. (1998). Evaluation of intestinal protozoan morphology in human fecal specimens preserved in EcoFix: comparison of Wheatley's trichrome stain and EcoStain. *Journal of Clinical Microbiology* **36**: 1974-1976.

Gardner, B.B., Del Junco, D.J., Fenn, J. & Hengesbaugh, J.H. (1980). Comparison of direct wet mount and trichrome staining techniques for detecting *Entamoeba* species trophozoites in stools. *Journal of Clinical Microbiology* **12**: 656-658.

Gonzalez-Ruiz, A., Haque, R., Aguirre, A., Castanon, G., Hall, A., Guhl, F., Ruiz-Palacios, G., Miles, M.A. & Warhurst, D.C. (1994). Value of microscopy in the diagnosis of dysentery associated with invasive *Entamoeba histolytica*. *Journal of Clinical Pathology* **47**: 236-239.

Haque, R., Mollah, N.U., Ali, I.K., Alam, K., Eubanks, A., Lyerly, D. & Petri, W.A., Jr. (2000). Diagnosis of amebic liver abscess and intestinal infection with the TechLab *Entamoeba histolytica* II antigen detection and antibody tests. *Journal of Clinical Microbiology* **38**: 3235-3239.

Huston, C.D. (2004). Parasite and host contributions to the pathogenesis of amebic colitis. *Trends in Parasitology* **20**: 23-26.

Huston, C.D., Haque, R. & Petri, W.A., Jr. (1999). Molecular-based diagnosis of *Entamoeba histolytica* infection. *Expert Reviews in Molecular Medicine* **1999**: 1-11.

Jackson, T.F. (1998). *Entamoeba histolytica* and *Entamoeba dispar* are distinct species; clinical, epidemiological and serological evidence. *International Journal for Parasitology* **28**: 181-186.

Koontz, F. & Weinstock, J.V. (1996). The approach to stool examination for parasites. *Gastroenterology Clinics of North America* **25**: 435-449.

Kuypers, F.A. (2007). Membrane lipid alterations in hemoglobinopathies. *Hematology American Society of Hematology Education Program* 68-73.

Lucas, R. & Upcroft, J.A. (2001). Clinical significance of the redefinition of the agent of amoebiasis. *Revista Latinoamericana de Microbiologia* **43:** 183-187.

Martinez-Palomo, A. (1982). *The Biology of Entamoeba histolytica*. Research Studies Press. A Division of John Wiley & Sons Ltd. pp. 1-59.

Mayer's. (2009). H & E staning Method and Protocol [Online] [Accessed 14/10/2009 9:19AM], Available from World Wide Web: http://www.ihcworld.com/_protocols/ special_stains/HE_Mayer.html

Mirelman, D., Monheit, D. & Varon, S. (1987). Inhibition of growth of *Entamoeba histolytica* by allicin, the active principle of garlic extract (Allium sativum). *Journal of Infectious Diseases* **156:** 243-244.

Nari, G.A., Ceballos Espinosa, R., Carrera Ladron de Guevara, S., Preciado Vargas, J., Cruz Valenciano, J.L., Briones Rivas, J.L., Moreno Hernandez, F. & Gongora Ortega, J. (2008). [Amebic liver abscess. Three years experience]. *Revista Española de Enfermedades Digestivas* **100:** 268-272.

SPI.Supplies. (2009). [Online] [Accessed 13/10/2009 5:18 PM], Available from World Wide Web: http://www.2spi.com/com/catalog/chem/Phloxine_B.shtml

Tanyuksel, M. & Petri, W.A. (2003). Laboratory diagnosis of amebiasis. *Clinical Microbiology Reviews* **16:** 713-729.

Visser, L.G., Verweij, J.J., Van Esbroeck, M., Edeling, W.M., Clerinx, J. & Polderman, A.M. (2006). Diagnostic methods for differentiation of *Entamoeba histolytica* and *Entamoeba dispar* in carriers: performance and clinical implications in a non-endemic setting. *International Journal of Medical Microbiology* **296:** 397-403.

WHO. (1997). Amoebiasis. *WHO Weekly Epidemiological Record* **72:** 97-100.

Zlobl, T.L. (2001). Amebiasis. *Primary Care Update for OB/GYNS* **8:** 65-68.

Tropical Biomedicine 26(3): 366–368 (2009)

Research Note

Seroprevalence of anti-amoebic antibody among blood donors by indirect hemeagglutination assay

Zeehaida, M.[1], Zairi, N.Z.[1], Tan, Z.N.[2], Wong, W.K.[2] and Lim, B.H.[2]
[1] Department of Medical Microbiology and Parasitology, School of Medical Sciences and
[2] School of Health Sciences, Universiti Sains Malaysia, Health Campus, 16150, Kubang Kerian, Kelantan
Email address: zeehaida@kck.usm.my
Received 21 September 2009; received in revised form 10 October 2009; accepted 12 October 2009

Abstract. The screening for anti-amoebic antibody among a group of donors was to obtain negative control serum samples for an on-going antigen development assay in diagnosis of amoebic liver abscess. Out of 200 samples, 125 (62.5%) were negative, whereas 44 (21.5%) had IHA titer of less than 1:128 and 31 (16.0%) of the samples had significant IHA titers of 1:128 or more, in which 2 serum samples gave titers of 1:4096.

The north-eastern state of peninsular Malaysia, Kelantan faces the South China Sea in the east and shares its border with Thailand in the north. It occupies an area of 15 020 sq. km and has a population of 1 478 800. Kelantan state is endemic for water-borne diseases, in which the overall incidence was less than 5 per 100 000 people from 2000 till 2004. The incidence of typhoid/paratyphoid in year 2004 was 1.87 per 100 000 population whereas the incidences of cholera, hepatitis A and dysentery were lower and accounted for 0.35, 0.42 and 0.43 per 100 000 population respectively (Ministry of Health Malaysia, 2004). Thus, according to the above figure, the estimated incidence of dysentery for this state is 6.36.

Though many pathogens cause dysentery, the prevalence of amoebiasis differs according to age, socioeconomic status and geographical distribution. There was no available data from previous publication on the background seropositivity of amoebiasis among healthy population in Malaysia. In Malaysia, blood donors are considered healthy adults aged more than 18 years, weighing more than 50 kg, clinically healthy and are seronegative for HIV, hepatitis B and C, syphilis and malaria. The screening for anti-amoebic antibody among this group of donors was to obtain negative control serum samples for an on-going antigen development assay in diagnosis of amoebic liver abscess. The serum samples were first screened by indirect hemagglutination assay (IHA), then followed by IHA titrations to select serum samples with titer of 1:64 or more, which were considered positive for amoebiasis, as suggested by the manufacturer (Dade-Behring Marburg, Germany).

Two hundred pooled serum samples from blood donors were screened by IHA; 125 (62.5%) were negative, whereas 44 (21.5%) and 31 (16.0%) had IHA titer of less than 1:128 and 1:128 or more titer respectively (Table 1). Detection of lower titers among blood donors could be due to previous exposure to amoebiasis among the local healthy population with either intestinal or extraintestinal forms. Thirty one

Table 1. Distribution of groups by IHA titers (n=200)

IHA titers	Number (percentage)
Negative	125 (62.5)
Less than 1:128	44 (21.5)
1:128 and more	31 (16.0)

Table 2. Distribution of IHA titers among the blood donors (n=200)

IHA titers	Number (percentage)
Negative	125 (62.5)
1:16	27 (13.5)
1:32	9 (4.5)
1:64	8 (4.0)
1:128	17 (8.5)
1:256	8 (4.5)
1:512	2 (1.0)
1:1024	2 (1.0)
1:2048	0 (0.0)
1:4096	2 (1.0)

(16.0%) of the samples had significant IHA titers of 1:128 or more, in which 2 serum samples gave titers of 1:4096 (Table 2). In these cases, the possibility of having some forms of amoebiasis at the time of blood donation could not be ruled out, neither the likelihood of being asymptomatic carriers.

In another report, the seroprevalence of villagers from West Kalimantan, Borneo who had IHA titers equal or greater than 1:128 was 7% (Cross *et al.*, 1976) as compared to this study which was 16.0%. The seroprevalence of blood donors from urban, suburban and rural population of Puebla State, Mexico which was done using IHA alone was 8.6% whereas when IHA and ELISA were employed together, as recommended by WHO, the seroprevalence was 6.4% (Sánchez-Guillén *et al.*, 2000).

Lower IHA titers had been demonstrated in patients with amoebic liver abscess (ALA) who were admitted to our hospital. In a previous study, 27.6% (16/58) ALA patients had antibody titer of 1:256 or less (Zeehaida *et al.*, 2008). The lower titers found in these patients could be due to low antibody levels in the early course of the disease.

The seroprevalence of amoebiasis among blood donors in this study was higher as compared to those reported previously in Malaysia and other surrounding endemic regions. The finding showed that the background seropositivity is significantly high among healthy population in this local setting. A lower titer of 1:128 could not be taken as a positive titer since it overlapped significantly with titers found among the blood donors. Thus, the titer had less value for diagnosis of extraintestinal amoebiasis. Supported by the clinical symptoms and signs of amoebiasis, 1:256 is deemed a significant titer for diagnosis of the disease, particularly in this local setting.

Acknowledgement. The study was partially funded by University Sains Malaysia Research University Grant, number: 1001/PPSK/813009

REFERENCES

Annual report, Ministry of Health Malaysia, 2004, page 88-89.

Cross, J.H., Clarke, M.D., Cole, W.C., Lien, J.C., Partono, F., Djakaria, Joesoef, A. & Oemijati, S. (1976). Parasitic infections in humans in West Kalimantan (Borneo), Indonesia. *Tropical Geography Medicine* **28(2)**: 121-30.

Sánchez-Guillén, M.C., Velázquez-Rojas, M., Salgado-Rosas, H., Torres-Rasgado, E., Pérez-Fuentes, R., Martínez-Munguía, J. & Talamás-Rohana, P. (2000). Seroprevalence of anti-*Entamoeba histolytica* antibodies by IHA and ELISA assays in blood donors from Puebla, Mexico. *Archives of Medical Research* **31** S53–S54.

Zeehaida, M., Wan Nor Amilah, W.A.W., Amry, A.R., Hassan, S., Sarimah, A. & Rahmah, N. (2008). A study on the usefulness of Techlab *Entamoeba histolytica* II antigen detection ELISA in the diagnosis of amoebic liver abscess (ALA) at Hospital Universiti Sains Malaysia (HUSM), Kelantan, Malaysia. *Tropical Biomedicine* **25**(3): 209–216.

Parasitol Res (2011) 108:425–430
DOI 10.1007/s00436-010-2083-8

ORIGINAL PAPER

Comparison of protein-free defined media, and effect of L-cysteine and ascorbic acid supplementation on viability of axenic *Entamoeba histolytica*

Weng Kin Wong · Zi Ning Tan · Boon Huat Lim · Zeehaida Mohamed · Alfonso Olivos-Garcia · Rahmah Noordin

Received: 29 June 2010 / Accepted: 8 September 2010 / Published online: 5 October 2010
© Springer-Verlag 2010

Abstract *Entamoeba histolytica* is the etiologic agent for amoebiasis. The excretory–secretory (ES) products of the trophozoites contain virulence factors and antigens useful for diagnostic applications. Contaminants from serum supplements and dead trophozoites impede analysis of ES. Therefore, a protein-free medium that can sustain maximum viability of *E. histolytica* trophozoites for the longest time duration will enable collection of contaminant-free and higher yield of ES products. In the present study, we compared the efficacy of four types of media in maintaining ≥95% trophozoite viability namely Roswell Memorial Park Institute (RPMI-1640), Dulbecco's Modified Eagle Medium (DMEM), phosphate-buffered saline for amoeba (PBS-A), and Hank's balanced salt solution (HBSS). Concurrently, the effect of adding L-cysteine and ascorbic acid (C&A) to each medium on the parasite viability was also compared. DMEM and RPMI 1640 showed higher viabilities as compared to PBS-A and HBSS. Only RPMI 1640 showed no statistical difference with the control medium for the first 4 h, however the ≥95% viability was only maintained for the first 2 h. The other protein-free media showed differences from the serum- and vitamin-free TYI-S-33 control media even after 1 h of incubation. When supplemented with C&A, all media were found to sustain higher trophozoite viabilities than those without the supplements. HBSS-C&A, DMEM-C&A, and RPMI 1640-C&A demonstrated no difference ($P>0.05$) in parasite viabilities when compared with the control medium throughout the 8-h incubation period. DMEM-C&A showed an eightfold increment in time duration of sustaining ≥95% parasite viability, i.e. 8 h, as compared to DMEM alone. Both RPMI 1640-C&A and HBSS-C&A revealed fourfold and threefold increments (i.e., 8 and 6 h, respectively) whereas PBS-A-C&A showed only onefold improvement (i.e., 2 h) as compared to the respective media without C&A. Thus, C&A-supplemented DMEM or RPMI are recommended for collection of ES products.

W. K. Wong
Institute for Research in Molecular Medicine (INFORMM),
Universiti Sains Malaysia,
16150 Kubang Kerian, Kelantan, Malaysia

R. Noordin (✉)
Institute for Research in Molecular Medicine (INFORMM),
Universiti Sains Malaysia,
11800 Penang, Malaysia
e-mail: rahmah8485@gmail.com

Z. N. Tan · B. H. Lim
School of Health Sciences, Universiti Sains Malaysia,
16150 Kubang Kerian, Kelantan, Malaysia

Z. Mohamed
Department of Medical Microbiology and Parasitology,
School of Medical Sciences, Universiti Sains Malaysia,
16150 Kubang Kerian, Kelantan, Malaysia

A. Olivos-Garcia
Departamento de Medicina Experimental, Facultad de Medicina,
Universidad Nacional Autónoma de México,
04510 México, Mexico DF, Mexico

Introduction

Entamoeba histolytica is an enteric protozoan parasite that causes amoebiasis. This disease affects more than 50 million people around the world and causes up to 100,000 fatal cases annually (Que and Reed 2000). This cosmopolitan

disease is common in human populations where poor sanitation and substandard personal hygiene prevail. The high prevalence areas include tropical and subtropical regions, like Mexico, Central and South America, India, South East Asia, Eastern and South Africa (Wells and Arguedas 2004). The transmission of amoebiasis is via fecal–oral route through the ingestion of infective stage cysts. Severity of the disease ranges from asymptomatic carrier to intestinal amoebiasis with symptoms that include amoebic colitis and dysentery, and the potentially fatal extra-intestinal amoebiasis caused by the haematogenous spread of active multiplying trophozoites to other organs. Amoebic liver abscess (ALA) is the most common manifestation of extra-intestinal amoebiasis (Petri and Singh 1999). Delay in diagnosis and subsequent treatment are the common causes of fatality in ALA cases (Akgun et al. 1999).

During active infection, *E. histolytica* trophozoites secrete and/or excrete products into the host environment. These excretory and secretory (ES) products contain virulence factors like amoebapores, cysteine proteases, collagenases, glycosidases, and other proteases that had been hypothesized to contribute to the pathogenesis of *E. histolytica* (Gitler et al. 1984; Guerrero-Manriquez et al. 1998; Debnath et al. 2005; Moncada et al. 2005). In addition, these ES products had also been shown to possess antigenic properties which are useful for diagnostic applications (Pal et al. 1996; Gupta et al. 1999; Sengupta et al. 2000). In order to collect the ES products, a protein-free maintenance medium is necessary to avoid "contamination" with non-parasite proteins and to maintain the viability of trophozoites. In addition, to facilitate reproducibility of the experiments, chemically defined medium would be preferred.

Previous studies have been performed to collect ES products by incubating *E. histolytica* trophozoites in various protein-free media, such as phosphate-buffered saline (PBS), Hank's balanced salt solution (HBSS), Dulbecco's Modified Eagle Medium (DMEM), and serum- and vitamin-free TYI-S-33 (Reed et al. 1989; Gupta et al. 1999; Moncada et al. 2005; Pal et al. 1996; Sengupta et al. 2000). These ES products were collected at the time when the viability of the trophozoites was at least 95%. The results showed that chemically non-defined TYI-S-33 medium was the most suitable to sustain ≥95% viability of trophozoites for up to 7 h. However the data in the above studies lacked statistical analysis to make convincing conclusions. Thus it is necessary that studies on media comparisons using appropriate statistics be performed to determine the best protein-free and chemically defined medium, for collection of *E. histolytica* ES products.

An early study by Diamond (1961) reported that 0.1% of L-cysteine and 0.02% ascorbic acid (C&A) could create an artificial anaerobic environment in axenic media for cultivation of *E. histolytica* (Dutta 1981). In the l studies by Gillin and Diamond (1980a, b), they have sh that the addition of C&A in maintenance medium cont ing bovine serum and vitamins can sustain the viabl *histolytica* for 12–24 h (Martinez-Palomo 1982).

In the present study, four types of protein-free chemically defined media commonly used in tissue cul were compared to determine the most suitable maintena medium for sustaining a minimum of 95% viability axenically grown *E. histolytica* trophozoites. Simultaneo the effect of supplementing the media with C&A was studied.

Materials and methods

Axenic culture of *Entamoeba histolytica*

The *E. histolytica* HM-1:IMSS axenic strain was use this study. The trophozoites were hermetically culture TYI-S-33 medium, containing 10% heat-inactivated bo serum (Gibco, New Zealand) and supplemented with Diamond vitamin (Sigma, USA), at 36°C (Diamond et 1978).

Preparation of protein-free maintenance media with/without C&A

The four types of media used in this experiment were follows: PBS for amoeba (PBS-A; 15 mM potassi phosphate and 175 mM sodium chloride, pH 7.0), HE without phenol red, pH 7.0, DMEM (Gibco, US Roswell Memorial Park Institute medium, No 1640 (RF 1640; Gibco, USA). All media were prepared us distilled water. PBS-A was autoclaved and HBSS filter-sterilized using 0.22-μm filter. In addition, all me with C&A supplementation were also prepared.

Viability study of *Entamoeba histolytica* in different protein-free media over time

A preliminary study was performed to estimate the lon time duration for survival of at least 95% of trophozoites in the four protein-free media. As the con trophozoites were maintained in serum- and vitamin- TYI-S-33 medium, i.e. undefined medium commonly u to grow *E. histolytica*. The morphology of the parasit each culture medium was observed and recorded until cells became rounded. For quantitative data anal viability of trophozoites was studied in RPMI 1640 m and DMEM for 8 h; and for up to 6 h in PBS-A and HE The duration of incubation was set based on prelimi observations.

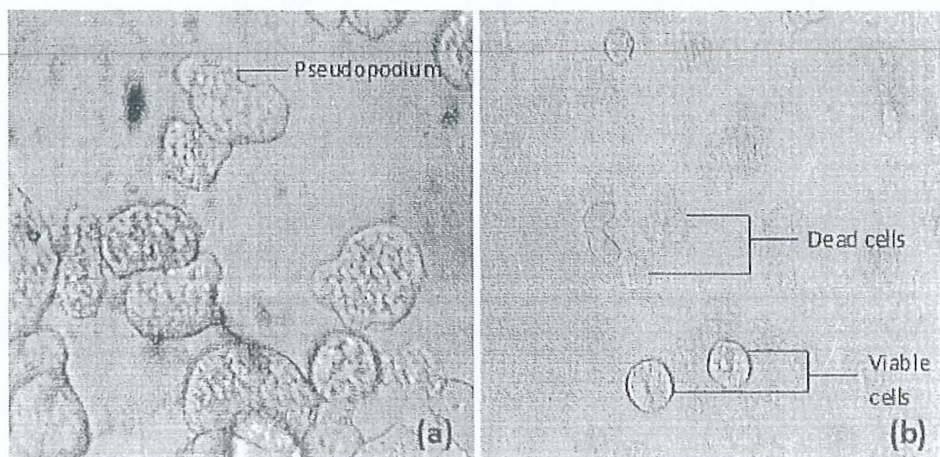


Fig. 1 Morphology of axenic *E. histolytica* trophozoites in PBS-A at magnification of 200× during **a** log phase and **b** after incubation for 6 h

The trophozoites used in the viability study were 48–72 h old, at which time they formed a monolayer on the wall of the culture tube containing the axenic TYI-S-33 medium. At each hourly interval, duplicate amoebic culture tubes containing each type of medium were examined. First, each tube was gently rinsed twice with 5 mL of a protein-free medium. The tube was then filled to the 80% level (10 mL) with the test medium. The initial viability of trophozoites was assumed to be 100% (Jimenez et al. 2004). At 1-h intervals, two tubes from each medium were chilled in crushed ice for 5 min, and then centrifuged at 500×*g* for 2 min. About 9 mL of supernatant was discarded. The pelleted trophozoites were gently mixed, and then the viability determined by Trypan blue exclusion method using Neubauer's chamber. The viability was expressed as mean of three separate experiments, each with two data replicates.

Statistical analysis

Least Significant Difference post hoc test was used to analyze the significant difference at hourly intervals between control medium and the protein-free media. A P value of <0.05 was considered as statistically different between the control media and the protein-free media.

Results

Morphology of *Entamoeba histolytica* after prolonged incubation in protein-free media

In the preliminary study performed using serum- and vitamin-free TYI-S-33 control medium, viable irregular-shaped trophozoites with amoeboid movements and pseudopodia attaching to the surfaces were observed throughout the 8-h incubation period. However, in the other media with or without C&A supplement, the trophozoites started to lose their viable characteristics (as described above) after 3 h. As the incubation time in the protein-free media increases, the cells started to become rounded. Figure 1 shows the changes in morphology when PBS-A was used as the medium.

Viability of *Entamoeba histolytica* in different protein-free medium over time

Figure 2 showed the comparisons among the protein-free defined media with or without C&A supplementation, and TYI-S-33 medium (serum- and vitamin-free) as the control. The *E. histolytica* trophozoites in the control medium consistently showed ≥95% viability throughout the 8-h incubation period. Without C&A supplementation, at the end of the 8-h incubation period, DMEM and RPMI 1640 showed higher parasite viabilities as compared to PBS-A and HBSS. Only RPMI 1640 showed no statistical difference in parasite viability as compared with the control medium for the first 4 h, however the ≥95% viability was only maintained at the first 2 h and decreased to 88% at 4 h. The other protein-free media without the supplements showed differences in parasite viabilities as compared with the control media even after 1 h of incubation.

With the addition of C&A supplements, DMEM-C&A showed a eightfold increment in parasite viability as compared to DMEM alone. Both RPMI 1640-C&A and HBSS-C&A revealed fourfold and threefold increments, respectively, whereas PBS-A-C&A showed only onefold improvement as compared to the respective media without C&A. Percentage viabilities of both DMEM-C&A and RPMI 1640-C&A were maintained at ≥95% throughout the 8-h incubation period. However, in HBSS-C&A medium, ≥95% trophozoites viability was sustained for only 6 h; then the percentage viability decreased to about 80%. In PBS-A-C&A, the percentage viability of the trophozoites were sustained at about 95% for the first 2-h incubation, but dropped to 87% (significantly lower than control medium, P<0.05) during the next hour.

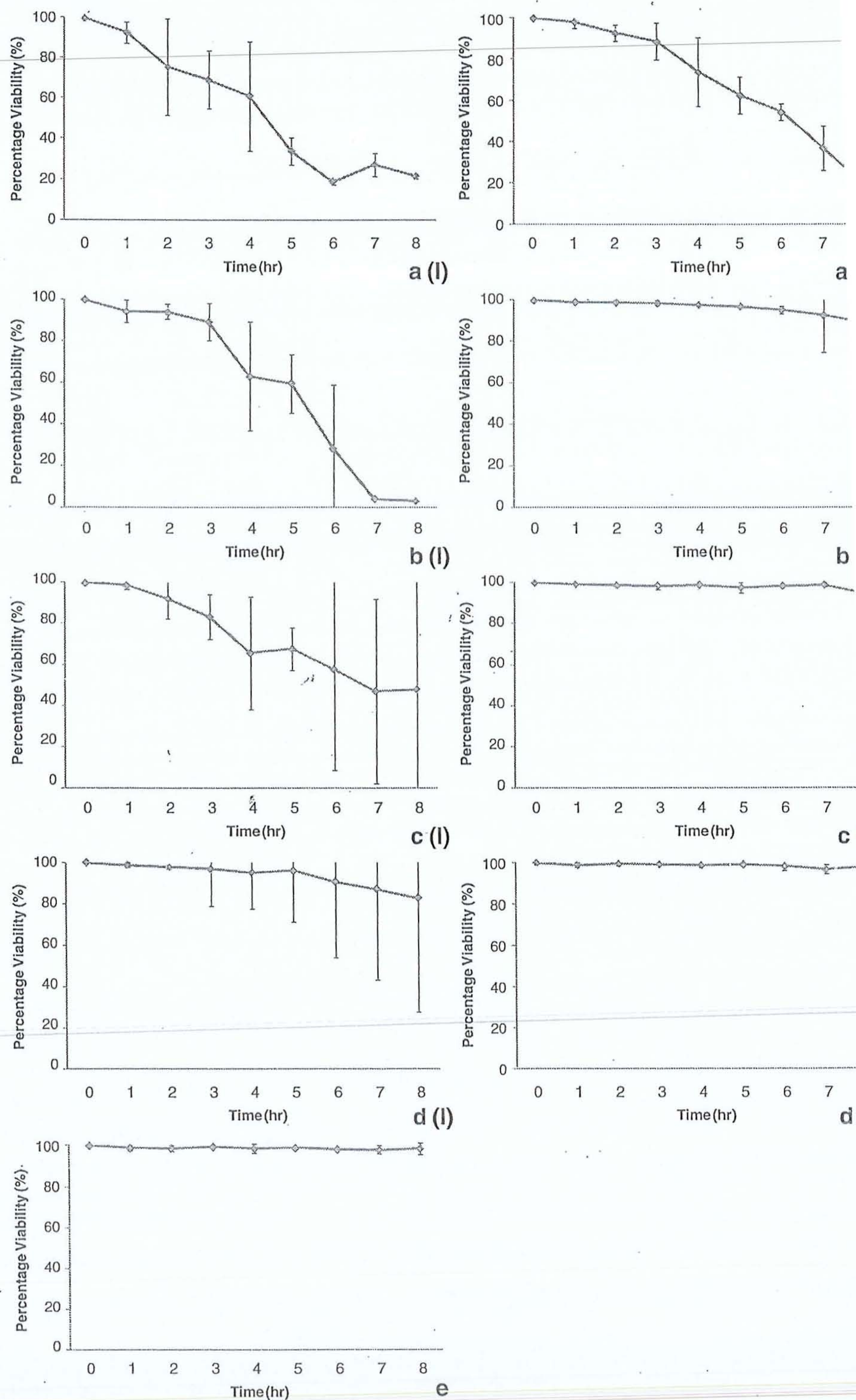


Fig. 2 Comparisons of percentage viabilities of *E. histolytica* trophozoites among the four protein-free media with/without C&A supplementation over time. Serum- and vitamin-free TYI-S-33 was the control medium. **a** (I), PBS-A; **a** (II), PBS-A-C&A; **b** (I), HBSS; **b** (II), HBSS-C&A; **c** (I), DMEM; **c** (II), DMEM-C&A; **d** (I), RPMI 1640; **d** (II), RPMI 1640-C&A; **e**, serum- and vitamin-free TYI-S-33

Discussion

Current ES collection methods could not exclude most of the proteins released from the trophozoites that lysed during the incubation process. Furthermore, Trypan blue exclusion method could only estimate the viability at the end of incubation period but could not estimate the trophozo that lysed during the process. Thus protein-free med that can prolong the viability of *E. histolytica* trophozo is pertinent in studies involving ES products. It will red

the amount of trophozoite proteins released from dead cells and the non-parasite contaminants from serum supplement. A protein-free and chemically defined medium will also facilitate reproducibility of the experimental data. Protein-free medium was also reported to be useful in immunological testing and functional studies such as interaction of *E. histolytica* with different cell lines (Guy et al. 1991).

In this study, viability of *E. histolytica* trophozoites in four different protein-free and chemically defined media suggested that among the media without supplementation, RPMI 1640 was the most suitable in sustaining ≥95% viability for the first 2 h and showed no statistical difference ($P<0.05$) with the control medium as compared with PBS-A, HBSS, and DMEM. With C&A supplementations, there were improvements in the general viability profiles over time in all media. This was especially evident by extension of the duration of ≥95% viability of trophozoites in PBS-A-C&A (onefold), HBSS-C&A (threefold), DMEM-C&A (eightfold), and RPMI 1640-C&A (fourfold). Similar result was also observed in the axenic culture of *Giardia lamblia*, another anaerobic intestinal protozoan. The RPMI 1640 medium supplemented with L-cysteine was reported to promote the viability and attachment of the parasite after prolonged incubation (Guy et al. 1991).

E. histolytica is an anaerobic protozoan that needs a low oxygen tension environment to grow (Sen et al. 2007). Band and Cirrito (1979) revealed that it was able to tolerate up to only 5% oxygen in culture media. In order to create an artificial anaerobic media for its survival, supplementation of 0.1% L-cysteine and 0.02% ascorbic acid into the axenic growth media (TYI-S-33 and TP-S-1) was introduced by Diamond (1961). With these supplements, the media was able to support the survival, as well as growth of *E. histolytica* trophozoites. Other reducing agents such as D-cysteine and thioglycolic acid have also been included into *E. histolytica* trophozoites culture for the same purpose (Gillin and Diamond 1980b). However, the combination of C&A was found to be the best for growth of trophozoites culture (Martinez-Palomo 1982). These supplements were reported to act as reducing agent as well as protective agent against oxidative stress. Later studies reported that good growth of trophozoites was also achieved using reducing agents like 0.2% L-cysteine and 0.2% reduced glutathione (Tekwani and Mehlotra 1999). However, thus far these supplements have not commonly been used in culture media.

Results from the present study were consistent with the earlier study by Gillin and Diamond (1980a, b), in which the maintenance medium supplemented with C&A was able to sustain the attachment, elongation, and amoeboid movement, as well as short-term survival of *E. histolytica* trophozoites. Similar report on cysteine supplementation in PBS (0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 20 mM cysteine) showed that it could sustain ≥95% viability of trophozoites for 3–4 h (Reed et al. 1989). This suggested that adding L-cysteine in PBS could improve the viability of trophozoites even in the absence of ascorbic acid.

There were obvious variations in the trophozoite viability periods when cultured in different media. In this study, ≥95% trophozoite viability was observed in PBS-A for less than 1 h, which is less than the 2 h reported by Sengupta et al. (2000) and 4 h reported by Gupta et al. (1999). In comparison to the results by Sengupta et al. (2000), the present study showed that HBSS sustained the same viability duration (2 h); but RPMI 1640 sustained an hour longer than the results in the former study.

In conclusion, among the four protein-free media used in this study, RPMI 1640 and HBSS could sustain ≥95% trophozoite viability for up to 2 h, hence are not suitable as maintenance media for prolonged incubation. However, ≥95% trophozoite viabilities were prolonged to 8 h with both DMEM-C&A and RPMI 1640-C&A; and 6 h with HBSS-C&A. In conclusion, this study showed that either DMEM or RPMI 1640 media supplemented with C&A were suitable for ES production since they could sustain ≥95% trophozoite viability for up to 8 h.

Acknowledgements This study was supported by Universiti Sains Malaysia Research University grant, no. 1001/PPSK/813009, FRGS grant, no. 203/CIPPM/6711122, and USM-RU-PRGS no. 1001/INFORMM/8032030. The first and second authors received financial support from USM Fellowship program.

References

Akgun Y, Tacyildiz IH, Celik Y (1999) Amebic liver abscess: changing trends over 20 years. World J Surg 23:102–106

Band RN, Cirrito H (1979) Growth response of axenic *Entamoeba histolytica* to hydrogen, carbon dioxide, and oxygen. J Protozool 26:282–286

Debnath A, Akbar MA, Mazumder A, Kumar S, Das P (2005) *Entamoeba histolytica*: characterization of human collagen type I and Ca^{2+} activated differentially expressed genes. Exp Parasitol 110:214–219

Diamond L (1961) Axenic cultivation of *Entamoeba histolytica*. Science 134: 336–337

Dutta GP (1981) Experimental and clinical studies on amoebiasis. McGraw-Hill, New Delhi

Gillin FD, Diamond LS (1980a) Attachment and short-term maintenance of motility and viability of *Entamoeba histolytica* in a defined medium. J Protozool 27:220–225

Gillin FD, Diamond LS (1980b) Attachment of *Entamoeba histolytica* to glass in a defined maintenance medium: specific requirement for cysteine and ascorbic acid. J Protozool 27:474–478

Gitler C, Calef E, Rosenberg I (1984) Cytopathogenicity of *Entamoeba histolytica*. Philos Trans R Soc Lond B Biol Sci 307:73–85

Guerrero-Manriquez GG, Sanchez-Ibarra F, Avila EE (1998) Inhibition of *Entamoeba histolytica* proteolytic activity by human salivary IgA antibodies. APMIS 106:1088–1094

Gupta S, Naik S, Naik SR (1999) Vaccine potential of 56–66 kDa protease secreted by *Entamoeba histolytica*. Indian J Med Res 109:141–146

Guy RA, Bertrand S, Faubert GM (1991) Modification of RPMI 1640 for use in vitro immunological studies of host-parasite interactions in giardiasis. J Clin Microbiol 29:627–629

Jimenez JC, Fontaine J, Grzych JM, Dei-Cas E, Capron M (2004) Systemic and mucosal responses to oral administration of excretory and secretory antigens from *Giardia intestinalis*. Clin Diagn Lab Immunol 11:152–160

Martinez-Palomo A (1982) The biology of *Entamoeba histolytica*. Research Studies Press, Chichester

Moncada D, Keller K, Chadee K (2005) *Entamoeba histolytica*-secreted products degrade colonic mucin oligosaccharides. Infect Immun 73:3790–3793

Pal S, Sengupta K, Manna B, Sarkar S, Bhattacharya S, Das P (1996) Comparative evaluation of somatic & excretory-secretory antigens of *Entamoeba histolytica* in serodiagnosis of human amoebiasis by ELISA. Indian J Med Res 104:152–156

Petri WA Jr, Singh U (1999) Diagnosis and management of ameb Clin Infect Dis 29:1117–1125

Que X, Reed SL (2000) Cysteine proteinases and the pathogene amebiasis. Clin Microbiol Rev 13:196–206

Reed SL, Keene WE, McKerrow JH (1989) Thiol prote expression and pathogenicity of *Entamoeba histolytica*. J Microbiol 27:2772–2777

Sen A, Chatterjee NS, Akbar MA, Nandi N, Das P (2007) Th kilodalton thiol-dependent peroxidase of *Entamoeba histo* is a factor involved in pathogenesis and survival of the pa during oxidative stress. Eukaryot Cell 6:664–673

Sengupta S, Akbar A, Mukhopadhyay P, Ganguly S, Sen P, D (2000) Role of excretory-secretory products of *Entam histolytica* in human amebiasis. Arch Med Res 31(4 Su S226–S228

Tekwani BL, Mehlotra RK (1999) Molecular basis of defence ag oxidative stress in *Entamoeba histolytica* and *Giardia lam* Microbes Infect 1:385–394

Wells CD, Arguedas M (2004) Amebic liver abscess. S Med J 97:673