Tropical Biomedicine 30(2): 250–256 (2013)

Efficacies of two in-house indirect ELISAs for diagnosis of amoebic liver abscess

Tan, Z.N.[1], Wong, W.K.[1], Noordin, R.[2], Zeehaida, M.[3], Olivos, G.A.[4] and Lim, B.H.[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
[*]Corresponding author e-mail: limbh493@gmail.com
Received 24 September 2013; received in revised form 28 February 2013; accepted 3 March 2013

Abstract. *Entamoeba histolytica* causes amoebic diarrhoea, colitis and liver abscess (ALA). Diagnosis of ALA is difficult, as most patients do not have simultaneous intestinal amoebic infection. At Hospital Universiti Sains Malaysia (HUSM), diagnosis of ALA relies on a combination of clinical findings, ultrasound examination of the liver and serodiagnosis using a commercial kit. In this study, two in-house indirect ELISAs were developed and evaluated. One of the in-house assays utilises *E. histolytica* crude soluble antigen (CSA) to detect serum IgG specific to the parasite whereas the other uses *E. histolytica* ether extract antigen (EEA). Preparation of CSA requires a sonicator to lyse the amoeba whereas EEA was prepared by chemically solubilizing the trophozoites. Based on the cut-off value of mean optical density + 3SD, CSA-ELISA showed 100% (24/24) sensitivity and 93.33% (210/225) specificity; while EEA-ELISA showed 91.67% (22/24) sensitivity and 95.11% (214/225) specificity. In conclusion, both the in-house indirect ELISAs were found to be efficacious for diagnosis of ALA; and the EEA is easier to prepare than the commonly used CSA.

INTRODUCTION

Amoebiasis causes between 40,000 to 100,000 deaths annually in the developing world (WHO, 1997); and the mortality is commonly due to amoebic liver abscess (ALA) (Haque *et al.*, 2010). Diagnosis of ALA is difficult, as most of the patients were either negative by stool microscopy or did not have blood in their stool (Katzenstein *et al.*, 1982). Molecular diagnosis based on identification of *E. histolytica* DNA in liver abscess pus is accurate, but the invasive aspiration of the sample may cause spillage of abscess contents (Pritt and Clark, 2008). Presence of anti-amoebic serum antibodies was reported to be unable to differentiate between present and past infections in the endemic areas (Abioye *et al.*, 1972; Thomas *et al.*, 1981). However, serodiagnosis of ALA does provide useful evidence on the existence of the infection, especially for patients from non-endemic areas. Interestingly, the current diagnosis for ALA is still based on aspiration of liver abscess combined with the detection of anti-amoebic serum antibodies (Haque *et al.*, 2010). Over the past 30 years, although numerous diagnostic assays were developed to diagnose ALA, serodiagnosis is still the preferred method in most diagnostic laboratories (Tanyuksel & Petri, 2003).

At Hospital Universiti Sains Malaysia (HUSM), IHA Cellognost® Amoebiasis Kit (Dade Behring Marburg GmbH, Germany) which detects anti-*E. histolytica* IgG serum antibodies is used for diagnosis of ALA at serum dilutions of 1:256 and higher (Zeehaida *et al.*, 2009). Although the commercial IHA

kit is reported to be sensitive, it would be more cost-effective if the laboratory could develop its own in-house assay that is at least as sensitive and specific as the commercial kit. Hence, in this study two in-house indirect-ELISAs were developed using *E. histolytica* crude soluble antigen (CSA) and *E. histolytica* ether extract antigen (EEA), subsequently they were evaluated using known positive and negative serum samples.

MATERIALS AND METHODS

Serum samples collection

This study was conducted in accordance with the requirement of Universiti Sains Malaysia Human Research Ethics Committee, USMKK/PPP/JEPeM (213.3.[10]). All the human serum samples were collected from HUSM since the year 2008 and kept at -20ºC until used. They comprised three categories of samples i.e. twenty four (24) serum samples from ALA patients, 28 from patients with other infections and 197 from blood donors.

Serum samples from patients with ALA

The 24 samples were collected from patients who were clinically diagnosed as ALA based on their clinical signs and symptoms; presence of liver abscess by ultrasound examination and high anti-amoebic titres by the commercial IHA kit.

Serum samples from patients with other infections

The 28 serum samples were from patients infected with pathogens other than *E. histolytica*. The causative pathogens were *enteropathogenic Escherichia coli* (n=1), *Shigella sonnei* Group D (n=1), *Salmonella* spp. (n=5), *Klebsiella pneumonia* (n=1), *Staphylococcus aureus* (n=1), *Ascaris lumbricoides* (n=1), *Escherichia coli* (n=2), coagulase-negative *Staphylococcus* (n=1), *Stenotrophomonas maltophilia* (n=1), *Helicobacter pylori* (n=1) and *Toxoplasma gondii* (n=9). Four other serum samples were obtained from patients with pyogenic liver abscess (PLA). All the serum samples tested negative by the commercial IHA kit.

Serum samples from blood donors

One hundred and ninety seven (197) blood donor serum samples were collected from HUSM Haematology & Blood Transfusion Unit, and tested negative by the commercial IHA kit.

Screening of serum samples with IHA

The IHA commercial kit was performed according to the manufacturer's instructions to screen for anti-amoebic antibodies in the serum samples. The cut-off value of 1:256 was used as an indicator for ALA, as reported in previous studies at HUSM (Zeehaida *et al*., 2008, 2009).

Preparation of ether extract antigen (EEA)

Ten million trophozoites were resuspended in 300 µL of phosphate-buffered saline specific for amoeba, PBS(A) followed by addition of 2X volume of diethyl-ether (Merck, Germany) and 20 µL of 0.5 M iodoacetamide (Sigma, USA). The mixture was then centrifuged at 110 x *g* for 5 min to separate the three layers i.e. the diethyl-ether layer (top layer), phospholipids layer (middle layer) and the soluble protein at the bottom. The bottom of the tube was then poked with a needle to create a tiny hole. The bottom layer was allowed to drip into a clean tube. The tube was placed in a fume hood to expedite the evaporation of excess diethyl-ether. It was then centrifuged at 10 000 x *g* for 30 min at 4°C. The supernatant containing the EEA protein was transferred into a clean tube and its protein concentration was estimated using Bio Rad Bradford protein assay (Bradford, 1976) before it was kept in -20ºC until used.

Preparation of crude soluble antigen (CSA)

Ten million *E. histolytica* trophozoites were mixed with 500 µL Complete Lysis-M buffer supplemented with a cocktail of protease inhibitor (Roche, Germany) and 20 µL of 0.5 M iodoacetamide (Sigma, USA). The cells were then disrupted with a sonicator (Branson, Mexico) at 10% amplitude for three cycles of 1 min sonication with 0.5 sec pulse-on and 0.5 sec pulse-off. The lysate was

centrifuged at 10 000 x *g* for 10 min at 4ºC to collect the CSA in the supernatant. After estimating the protein concentration with Bradford protein assay (Bradford, 1976), the CSA kept in -20ºC until used.

Comparison of protein profiles of CSA and EEA

Protein profiles of both CSA and EEA were compared by loading 10 µg of each protein into each well of a 12% polyacrylamide gel. The electrophoretic separation was run at a constant current of 25 mA per gel until the bromophenol blue dye was ~0.5 cm from the bottom of the glass plate. The gel was stained with Coomassie brilliant blue (CBB) (Bio Basic, Canada) for 30 min and destained until the background of the gel was clear. The CSA and EEA protein profiles on the gel were then captured with a scanner (All in One Brother DCP-165C Printer, USA).

Indirect Enzyme-linked immunosorbent assay (Indirect-ELISA)

The indirect-ELISAs were performed as described by Reen (1994) with some modifications. The optimized coating concentrations of CSA and EEA per microtiter well (NUNC, Denmark) were 20 µg/mL and 40 µg/mL, respectively. The optimized primary and secondary antibody dilutions were 1:50 and 1:500 respectively for both ELISAs. Briefly, each antigen was diluted in 0.1 M bicarbonate buffer (pH 9.6), then 100 µL was pippeted into each well. The microtiter plate was then incubated in a moisture chamber overnight at 4ºC. The wells were then washed (3 x 5 min) with 200 µL phosphate-buffer saline (PBS) containing 0.05% Tween 20 (PBST). The wells were blocked with 200 µL blocking reagent (Roche, Germany) for 1 h at room temperature. The wells were again washed 3 x 5min with PBST, then 100 µL of 1:50 dilution human serum was added into each well and incubated for 1 hour at room temperature. After a washing step, each well was incubated with 100 µL of 1:500 dilution of horse radish peroxidase (HRP)-conjugated mouse monoclonal anti-human antibody (Invitrogen, USA) for 1 h at room temperature. After another washing step, 100 µL tetramethylbenzidine (TMB) solution was added and incubated for 15 min at room temperature. The results were finally read at 450nm using VERSAmax^{TM} Microplate Reader (Sunnyvale, USA). The COV for each indirect-ELISA was calculated based on the mean optical density (OD) values plus three standard deviations (SD) of the ELISA results of the 197 blood donor samples.

Determination of the efficacies of CSA-ELISA and EEA-ELISA

The sensitivity rate of each assay was calculated based on the results obtained using the 24 ALA patients serum samples. Meanwhile the specificity rate of each assay was calculated based on the results obtained using the 28 serum samples from patients with other infections and 197 blood donor serum samples.

Statistical analysis

Statistical analysis was performed using SPSS for Window version 19.0. Comparisons were made between the sensitivities and specificities of each of the CSA-ELISA and EEA-ELISA using chi-square test; if indicated Fisher's exact test was used instead.

RESULTS

Comparison of Protein Profiles of CSA and EEA

The protein profiles on 12% SDS-PAGE showed that both EEA and CSA shared many distinct protein bands such as ~100 kDa, ~45 kDa and ~38 kDa. In addition, two protein bands at ~40 kDa and ~32 kDa were observed to be present in CSA but not in EEA protein profile (Figure 1).

COVs of CSA-ELISA and EEA-ELISA

The COV of CSA-ELISA at mean OD value + 3SD was determined to be 1.08; while that of EEA-ELISA was 0.76.

Sensitivities and Specificities of CSA-ELISA and EEA-ELISA

CSA-ELISA was found to be 100% (24/24) sensitive and 93.33% (210/225) specific, whereas EEA-ELISA showed a sensitivity

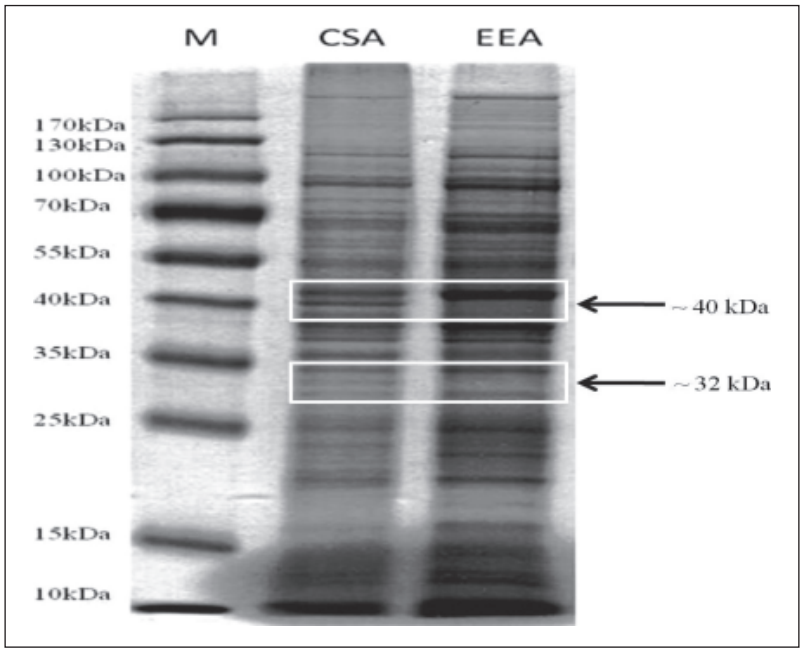

Figure 1. Protein profiles of CSA and EEA on 12% SDS-PAGE; M: PageRuler™ Prestained Protein Ladder (Fermentas); The two boxes indicate the protein bands observed in CSA but absent in EEA

rate of 91.67% (22/24) and a specificity rate of 95.11% (214/225) (Tables I and II).

Based on the statistical analysis, there were no significant different in terms of sensitivity ($p < 0.05$) between CSA-ELISA and EEA-ELISA [Table III(A)], however both ELISAs were significantly different ($p < 0.05$) in terms of specificity in the diagnosis of ALA [Table III(B)].

DISCUSSION

Invasive amoebiasis such as ALA could be life-threatening if prompt diagnosis and treatment is not given. Among the wide range of available serological diagnostic methods, IHA is one of the promising assays, as its sensitivity was reported to be in the range of 90% to 100% (Salles, 2003). However, the commercial kit is rather costly and not suitable for routine screening of invasive amoebiasis in low-resource endemic areas. Thus, cheaper but valid and reliable in-house diagnostic assay which is comparable to IHA should be developed for use in the endemic areas.

ELISA is a quantitative assay which is suitable for routine diagnostic laboratory due to its simplicity, sensitivity and reliability. In

Table 1. Results of three categories of serum samples tested using CSA-ELISA

CSA-ELISA (COV= mean+3SD)	Number of Serum samples		Total
	< 1.08	≥ 1.08	
Human ALA serum	0	24	24
Other infections human serum	28	0	28
Blood donors' serum	182	15	197
Total	210	39	**249**

Sensitivity = 100% (24/24)
Specificity = 93.33% (210/225)

Table 2. Results of three categories of serum samples tested using EEA-ELISA

EEA-ELISA (COV= mean+3SD)	Number of Serum samples		Total
	< 0.76	≥ 0.76	
Human ALA serum	2	22	24
Other infections human serum	26	2	28
Blood donors' serum	188	9	197
Total	216	33	**249**

Sensitivity = 91.67% (22/24)
Specificity = 95.11% (214/225)

Table 3. Comparison between CSA-ELISA and EEA-ELISA in terms of sensitivity and specificity in diagnosis of ALA

(A)

Sensitivity		EEA-ELISA		Total	Fisher's Exact Test (p-value)
		.00	1.00		
CSA-ELISA	1.00	2	22	24	*
Total		2	22	24	

*No statistics are computed because CSA is a constant

(B)

Specificity		EEA-ELISA		Total	Fisher's Exact Test (p-value)
		.00	1.00		
CSA-ELISA	.00	205	5	210	.000
	1.00	9	6	15	
Total		214	11	225	

Note: (.00): Negative; (1.00): Positive
$p<0.05$ indicates significant difference between the two variables

this study, in-house indirect-ELISAs utilising two different antigen preparations (i.e. CSA and EEA) were developed and subsequently tested for their sensitivities and specificities for serodiagnosis of ALA. Das *et al.* (1979) was among the pioneers who introduced the standard amoebic lysate antigen preparation using sonication technique (equivalent to CSA) for serodiagnosis and seroepidemiological study of amoebiasis. In the present study CSA-ELISA was found to be suitable for diagnosis of ALA based on its high sensitivity and specificity, and is comparable to the efficacy of the commercial IHA kit. Over 30 years, most of the antigens employed in indirect-ELISA were either *E. histolytica* CSA, purified lectin antigen or recombinant proteins (Lin *et al.*, 1981; Pal *et al.*, 1996; Stanley *et al.*, 1998; Abd-Alla *et al.*, 2000; del Carmen Sanchez-Guillen *et al.*, 2000; Haque *et al.*, 2000; Haghighi & Rezaeian, 2005; van Doorn *et al.*, 2005).

The most interesting finding of this study is the highly promising efficacy of EEA for diagnosis of ALA. To our knowledge, this is the first report on the use of EEA as a diagnostic reagent. Both the sensitivity and specificity rates of EEA-ELISA exceeded 90%, which are on par with the efficacy of the commonly used CSA-based ELISA for diagnosis of ALA (Yang & Kennedy, 1979; Tachibana *et al.*, 2004). Hence, EEA has the potential to be an alternative diagnostic antigen for the serodiagnosis of ALA.

Harvesting CSA is tedious, as it requires sonication to obtain the soluble proteins; whereas preparation of EEA requires only diethyl-ether to remove the integral membrane proteins in the organic phase and it contains only the intracellular proteins (Warnecke & Heinz, 1994).

In comparing the Coomassie blue-stained protein profiles of EEA and CSA, there were no major observable differences in terms of number and intensity of bands, although the ~40 kDa and ~32 kDa protein bands in the EEA profile seemed to be absent in the CSA profile. However this needs further studies since the apparent absence of the two protein bands could be due to the low sensitivity of the Coomassie blue stain.

Interestingly both the specificity rates of EEA-ELISA (95.11%) and CSA-ELISA (93.33%) for diagnosis of ALA exceeded 90%, but they were found to be statistically different. Thus future studies should focus on in-depth comparison of the two antigens in order to identify the common and unique antigenic protein(s) of EEA and CSA, and subsequently utilise it/them to improve on the efficacy of serodiagnosis of ALA. Ideally, the newly discovered antigen(s) could be developed into a rapid diagnostic test which can diagnose ALA from a small volume of finger-prick blood.

Acknowledgements. This study was funded by Universiti Sains Malaysia Research University Grant: 1001/PPSK/813044. TZN and WWK, the first two authors received financial support through the university fellowship. We thank the Dean of School of Health Sciences, Universiti Sains Malaysia for the facilities made available in the Biomedicine Laboratory.

REFERENCES

Abd-Alla, M.D., Jackson, T.F., Reddy, S. & Ravdin, J.I. (2000). Diagnosis of invasive amebiasis by enzyme-linked immunosorbent assay of saliva to detect amebic lectin antigen and anti-lectin immunoglobulin G antibodies. *Journal of Clinical Microbiology* **38**(6): 2344-2347.

Abioye, A.A., Lewis, E.A. & McFarlane, H. (1972). Clinical evaluation of serum immunoglobulin amoebiasis. *Immunology* **23**: 937.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**: 248-254.

Das, S.R., Kidwai, S.A. & Gupta, A.K. (1979). Preparation of standard amoeba-antigen from axenic *Entamoeba histolytica* and its use in the serodiagnosis and seroepidemiology of amoebiasis. *Journal of Biosciences* **1**(3): 255-261.

del Carmen Sanchez-Guillen, M., Velazquez-Rojas, M., Salgado-Rosas, H., Torres-Rasgado, E., Perez-Fuentes, R., Martinez-Munguia, J. & Talamas-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**(4 Suppl): S53-4.

Haghighi, A. & Rezaeian, M. (2005). Detection of serum antibody to *Entameba histolytica* in various population samples of amebic infection using an enzyme-linked immunosorbent assay. *Parasitology Research* **97**(3): 209-212.

Haque, R., Kabir, M., Noor, Z., Rahman, S.M.Z., Mondal, D., Alam, F., Rahman, I., Mahmood, A.A., Ahmed, N. & Petri, W.A., Jr. (2010). Diagnosis of amebic liver abscess and amebic colitis by detection of *Entamoeba histolytica* DNA in blood, urine, and saliva by a real-time PCR assay. *Journal of Clinical Microbiology* **48**: 2798-2801.

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**(9): 3235-3239.

Katzenstein, D., Rickerson, V. & Braude, A. (1982). New concepts of amebic liver abscess derived from hepatic imaging, serodiagnosis, and hepatic enzymes in 67 consecutive cases in San Diego. *Medicine* **61**: 237-246.

Lin, T.M., Halbert, S.P., Chiu, C.T. & Zarco, R. (1981). Simple standardized enzyme-linked immunosorbent assay for human antibodies to *Entamoeba histolytica*. *Journal of Clinical Microbiology* **13** (4): 646-651.

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 Journal of Medical Research* **104:** 152-156.

Pritt, B.S. & Clark, C.G. (2008). Amebiasis. Mayo Clinic Proceedings **83**(10): 1154-1159.

Reen, D.J. (1994) Basic Protein and Peptide Protocols: CHAPTER 47 Enzyme-Linked immunosorbent Assay (ELISA), Humana Press Inc., Totowa, NJ.

Salles, J.M., Moraes, L.A. & Salles, M.C. (2003). Hepatic amebiasis. *Brazilian Journal of Infectious Diseases* **7**(2): 96-110.

Stanley, S.L., Jr., Jackson, T.F., Foster, L. & Singh, S. (1998). Longitudinal study of the antibody response to recombinant *Entamoeba histolytica* antigens in patients with amebic liver abscess. *American Journal of Tropical Medicine and Hygiene* **58**(4): 414-416.

Tachibana, H., Cheng, X.J., Masuda, G., Horiki, N. & Takeuchi, T. (2004). Evaluation of recombinant fragments of *Entamoeba histolytica* Gal/GalNAc lectin intermediate subunit for serodiagnosis of amebiasis. *Journal of Clinical Microbiology* **42**(3): 1069-1074.

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

Thomas, V., Sinniah, B. & Leng, Y.P. (1981). Assessment of the sensitivity, specificity, and reproducibility of the indirect immunofluorescent technique for the diagnosis of amebiasis. *American Journal of Tropical Medicine and Hygiene* **30**: 57-62.

van Doorn, H.R., Hofwegen, H., Koelewijn, R., Gilis, H., Peek, R., Wetsteyn, J.C., van Genderen, P.J., Vervoort, T. & van Gool, T. (2005). Use of rapid dipstick and latex agglutination tests and enzyme-linked immunosorbent assay for serodiagnosis of amebic liver abscess, amebic colitis, and *Entamoeba histolytica* cyst passage. *Journal of Clinical Microbiology* **43**(9): 4801-4806.

Warnecke, D.C. & Heinz, E. (1994). Purification of a membrane-bound UDP-glucose:sterol [beta]-D-Glucosyl-transferase based on its solubility in diethyl ether. *Plant Physiology* **105**(4): 1067-1073.

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

Yang, J. & Kennedy, M.T. (1979). Evaluation of enzyme-linked immunosorbent assay for the serodiagnosis of amebiasis. *Journal of Clinical Microbiology* **10**(6): 778-785.

Zeehaida, M., Wan Nor Amilah, W.A., 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.

Zeehaida, M., Zairi, N. Z., Tan, Z.N., Wong, W.K. & Lim, B.H. (2009). Seroprevalence of anti-amoebic antibody among blood donors by indirect hemeagglutination assay. *Tropical Biomedicine* **26**(3): 366-368.