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

Running title: *Entamoeba histolytica* ESA of diagnostic potential

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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.
Amoebiasis is caused by the enteric protozoan *Entamoeba histolytica*, which affects 50 million of the world population and leads to 100,000 fatal cases annually (19, 20). Amoebic liver abscess (ALA) is the most common clinical manifestation of extraintestinal amoebiasis. It is due to the haematogenous spread of the *E. histolytica* trophozoites from intestine to liver through the portal vein. Patients with ALA present with hepatomegaly, right upper quadrant pain, tenderness of the liver, fever, jaundice and nausea. It may lead to fatal outcome if early diagnosis and treatment are not sought (1, 10).

Diagnosis of ALA is often initiated with radiology imaging to examine the presence of abscess in the liver. If indicated, aspiration of the sample is performed for culture, DNA detection and/or antigen detection. The indications include large abscesses, superficial abscesses, abscesses with severe pain or marked point tenderness, abscesses with marked diaphragm elevation, clinical picture suggesting impending abscess perforation and left lobe abscess (7). Absence of bacteria growth in the abscess culture could rule out the possibility of pyogenic liver abscess cases. The definitive diagnosis of ALA is by microscopic observation of trophozoites in the abscess fluid, but the sensitivity of microscopic examination is low as the trophozoites are easily disintegrated and most of them reside at the peripheral margin of the abscess. Many reports showed that DNA and antigen detection-based methods performed on the abscess sample *e.g.* PCR, real-time PCR, Techlab *E. histolytica* II antigen detection ELISA, gave high sensitivity (4, 11, 18).
Besides imaging, serological test is the preferred choice for diagnosis. The available antigen detection tests such as Techlab *E. histolytica* II ELISA, which detects *E. histolytica* lectin antigen, can be used for diagnosis of acute ALA patient who have not received treatment (23). However often patients who are admitted to the hospital with liver abscess have received treatment prior to investigation for ALA, which significantly reduces the sensitivity of the antigen detection test. Thus antibody detection is currently the most common serological test used to detect ALA, either by indirect haemagglutination assay (IHA) or ELISA. However, these tests mostly use amoebic lysate antigen, and are problematic for diagnosis in endemic area where the background anti-amoebic antibody titer is high. Thus in endemic areas, low specificities of these tests were reported with the low cut-off values as suggested by the manufacturer (22, 24).

Comparison of crude soluble antigen (CSA) with excretory secretory antigen (ESA) of *E. histolytica* have been shown to demonstrate higher positive detection rate when tested with sera of patients with acute amoebic dysentery and asymptomatic cysts passer, and equal sensitivity for diagnosis of ALA (10, 15). Therefore, in our quest to identify new markers to improve the serodiagnosis of ALA, ESA of *E. histolytica* was produced and analysed by SDS-PAGE, two-dimensional electrophoresis (2-DE) and Western blot. The identities of the potential candidates were then identified by mass-spectrometry.
MATERIALS AND METHODS

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

Collection and preparation of ESA. Mass culture of *E. histolytica* trophozoites were collected at log phase and washed 3X with RPMI supplemented with 0.1 % L-cysteine and 0.02 % ascorbic acid (RPMI-C&A) with centrifugation at 22 x g for 2 min, RT. Subsequently, the cell density was determined via trypan blue exclusion method. Trophozoites were seeded into culture tube containing 80 % filled RPMI-C&A medium with cell density of 0.8 x 10^6 cells per mL and incubated at 36 °C for 6 hours. Using this method, we have previously shown that ~95% trophozoites viability can be maintained throughout the incubation period (21). Upon completion, culture tubes were subjected to centrifugation at 22 x g for 2 min at 4 °C. The supernatant in the culture tubes were collected and mixed with 0.5 M iodoacetamide to a final concentration of 1 mM. Next, the supernatant was again subjected to centrifugation at 10,000 x g for 5 min at 4 °C and filtered through 0.2 µm membrane. Subsequently, ESA in the supernatant was concentrated 1000X using U-Tube concentrator with MWCO of 10 kDa. Cocktail protease inhibitor (Roche Diagnostic, Germany) was then added to the concentrated ESA. The protein concentration of the ESA was estimated using Bradford protein assay (10, 15, 21).
Serum samples and ethical approval. Serum samples in the current study were collected from Hospital Universiti Sains Malaysia during the period of 2008-2010. The procedures of collecting and handling the serum samples were approved by USM Human Ethical Committee (Ref. No.: USMKK/PPP/JEPem[213.3(10)]). Human serum samples included in this study were divided into four groups: (i) Group A: human ALA serum samples (n=24) with consistent clinical symptoms (i.e. fever, abdominal/right hepatic chest pain, hepatomegaly, and jaundice) and radiological image, and IHA positive results; (ii) Group B: human ALA serum samples (n=14) from patients whose abscess were positive by real-time PCR for E. histolytica DNA and negative by bacterial culture, the primers and probe sequences were as reported previously (9); (iii) Group C: healthy blood donor serum samples which were negative by IHA (n=30); (iv) Group D: serum samples from patients with other infections (n=33) i.e. salmonellosis (n=5), shigellosis (n=1), Escherichia coli septicaemia (n=2), Staphylococcus spp. septicaemia (n=2), H. pylori (n=6), pyogenic liver abscess (n=4), Stenotrophomonas maltophilia septicaemia (n=1), enteropathogenic Escherichia coli (n=1), Ascaris lumbricoides (n=1), Klebsiella pneumoniae (n=1) and toxoplasmosis (n=9). These sera were negative by IHA for amoebiasis.

SDS-PAGE. Protein samples were electrophoretically separated via SDS-PAGE using Bio-Rad Mini Protean III Electrophoresis Cell and Protein® II xi Cell according to Laemmli (8) protocol with modifications. Prior to SDS-PAGE, ESA was mixed with 2X Laemmli sample buffer and boiled for 5 min. Subsequently, it was separated using 6% SDS-PAGE gel, at constant current of 25 mA per gel for about 1 h.
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 1 hr 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 human sera at dilution of 1:200 (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–human IgG conjugated with horseradish peroxides (HRP) at dilution of 1:6000 for 1 hr. 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-DE and Western blot. Selected protein bands which showed potential diagnostic value were further analysed using 2-DE to ensure that the bands were well-separated. OFFGEL fractionator 3100 (Agilent Technologies, Germany) was used to separate the proteins by isoelectric points (pI) followed by SDS-PAGE and Western blot. Agilent OFFGEL Kit pH 3–10 with a 12-well setup frame was used. Sample was prepared by mixing 1600 µl of the ready stock solution (1.25X) with 400 µl of the sample with total protein amount of 2 mg and then gently vortexed. Forty microliter of IPG strip rehydration buffer was added into each well to swell the gel for 15 minutes. Wetted electrode pads were placed at the cathode- and anode-ends of the IPG strip gel surface.
After re-swelling of the gel, 150 µL of protein sample was loaded into each well. Ten
microliter of rehydration buffer was reapplied onto the electrode pads at each of the IPG
gel ends. Cover fluid (mineral oil) was pipetted onto the gel strip ends. Subsequently, the
sample was focused with a maximum power of 200 mW, maximum current of 50 mA and
typical voltages ranging from 500 to 4500 V until 50 kVh was reached after 24 h. Upon
completion, each of the twelve fractionated ESA samples were separately mixed with 5X
Laemmli sample buffer without boiling and electrophoretically separated via SDS-
PAGE. Western blot was performed using pooled and individual human serum samples to
identify the selected antigenic proteins.

Mass spectrometry analysis & protein Identification. The selected proteins
were excised from 2D-SDS-PAGE gel and sent for MALDI-TOF-TOF (4800) analysis at
Proteomic Laboratory Service Center, Australia, and searched with Swiss-Prot protein
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 above 80 % (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. In this study, sensitivity is defined as the number of serum samples which reacted with the band out of the total number of samples from ALA patients, i.e., Group A and B sera. Meanwhile, specificity is defined as the number of serum samples which do not react with the band out of the total number of samples from individuals without ALA, i.e., Group C and D sera.

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 (C4LTM0) 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. Since pyruvate phosphate dikinase has not been reported as a diagnostic protein, the 110 kDa band from separate gels were sent three times for the MALDI-TOF-TOF analysis, and same result was obtained each time.
DISCUSSION

Current diagnosis of ALA still depends on the results of clinical manifestations, radiology imaging and serology, since stool examination is inapplicable for diagnosis of extraintestinal amoebiasis and abscess aspiration is only performed when indicated.

E. histolytica ESA contains proteins shed from trophozoites during active multiplication and metabolites released by trophozoites during incubation in RPMI-C&A. Although great care was taken to produce good quality ESA, there were probably still some partial proteins released from lysed trophozoites. In this study, the ESA antigenic bands that ranged from 97.2-158 kDa consistently showed reactivities when incubated with human ALA serum samples (n=7). Analysis of the IgG blots showed that 152 kDa and 110 kDa proteins had higher association with serum samples from patients with PCR-positive abscess i.e. 13/14 (93 %) and 12/14 (86 %) respectively, as compared to serum samples from patients with unknown PCR results i.e. 19/24 (79 %) for both bands. Besides, the sensitivity of both 152 kDa and 110 kDa were similar, i.e. 32/38 (84 %) and 31/38 (82%) respectively. Both antigens showed high specificity as there were undetectable reactivities in the IgG blot of ESA probed with serum samples from normal individuals and those with other infections.

The protein components of ESA in the current study were different from those reported by Sengupta et al.(15). In the latter study, the ESA proteins ranged between 200-20 kDa, with predominant protein bands below 66 kDa (e.g. 45 kDa and 29 kDa), while the high molecular weight proteins (> 100 kDa) were faint. This may be due to the
differences in antigen preparations. The ESA in this study was concentrated 1000X instead of 10X to enrich the low abundant proteins. In addition, to enhance the reproducibility of ESA, protein-free defined RPMI-C&A was used in this study, instead of serum- and vitamin-free TYI-S-33. Besides, iodoacetamide was added to the RPMI-C&A containing the ESA upon its collection, in order to protect the protein from degradation by proteases (3, 13, 15). Although we have previously optimised the growth conditions of *E. histolytica* trophozoites at which ~95% of the parasite was alive when ESA was collected (21), nevertheless it is still possible that some of the antigenic bands may have come from ruptured trophozoites.

2-DE protein separation *via* Agilent 3100 OFFGEL Fractionator followed by SDS-PAGE allowed only the selected ESA fractions to be tested with serum samples. The protein bands excised from the 2-DE gels were well-separated, thus avoiding the presence of multiple proteins in each band. In this study, the 152 kDa protein was identified as *E. histolytica* lectin protein, which has been reported to be sensitive for diagnosis of invasive amoebiasis (5, 6). Specific monoclonal antibody against this protein has been used for antigen detection test in TechLab Entamoeba histolytica II kit (TechLab Inc, USA). The 110 kDa protein, identified as the *E. histolytica* pyruvate phosphate dikinase, was also found to show similar high sensitivity for diagnosis of ALA. This protein was reported to be a key enzyme in the anaerobic metabolism *via* pyrophosphate dependent glycolysis, and has no counterpart with proteins in human metabolism (14). Molecular modeling of this enzyme had been reported, and specific inhibitors to it for therapeutic purpose have been studied (17). The protein sequence of
pyruvate phosphate dikinase showed high similarity with a closely related pathogenic intestinal anaerobic protozoa, *Giardia lamblia*. This suggests the possibility of producing specific antibody to pyruvate phosphate dikinase for simultaneous detection of both *E. histolytica* and *G. lamblia* species in faecal samples (14).

To date, there is no report on the application of pyruvate phosphate kinase for diagnosis of amoebiasis. Nevertheless, to confirm the diagnostic value of pyruvate phosphate dikinase, it would be necessary to produce the recombinant form of the protein, then demonstrate that is recognized by patients’ sera. This is indeed our aim for future research.

**ACKNOWLEDGEMENTS**

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Figure 1  Representative IgG blot of E. coli probed with human serum samples. Lanes 1-7: individual AIA serum samples from Group A; Lane 8: pooled IHA negative serum sample (Group C); Lane 9: pooled AIA serum sample (positive control); Lane 10: TBS
Figure 2 Representative IgG blot of ESA when probed with human serum samples.

1. Lane 1: TIB; Lane 2: pooled ALA serum sample (positive control); Lane 3: pooled BIA
2. negative serum sample (Group D); Lanes 4-10: individual ALA serum samples from
3. Group B.
Table 1  Sensitivity and specificity of antigenic bands of ESA from IgG blots

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<th>Antigenic band, kDa</th>
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<td>152</td>
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<td>76</td>
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Sensitivity: Number of positive tests within the groups with ALA i.e. Groups A and B

Specificity: Number of negative tests within the groups without ALA i.e. Groups C and D
Figure 3  IgG blot of ISA separated by 7-GE, and probed with human serum
samples. H+: pooled positive ALA serum sample from Group B; H-: pooled HIA
negative serum sample (Group C).